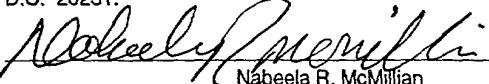


PATENT APPLICATION

ARCD:272

APPLICATION FOR UNITED STATES LETTERS PATENT  
for  
MUTATIONS IN THE DIABETES SUSCEPTIBILITY GENES  
HEPATOCYTE NUCLEAR FACTOR (HNF) 1 ALPHA ( $\alpha$ ), HNF-1 $\beta$  AND HNF-4 $\alpha$   
by  
Graeme I. Bell  
Kazuya Yamagata  
Naohisha Oda  
Pamela J. Kaisaki  
Hirotomo Furuta  
Yukio Horikawa  
and  
Stephen Menzel

EXPRESS MAIL MAILING LABEL	
NUMBER	EM423824718US
DATE OF DEPOSIT	September 9, 1997
I hereby certify that this paper or fee is being deposited with the United States Postal Service "EXPRESS MAIL POST OFFICE TO ADDRESSEE" service under 37 C.F.R. 1.10 on the date indicated above and is addressed to: Assistant Commissioner for Patents, Washington D.C. 20231.	
 Nabeela R. McMillian	

## BACKGROUND OF THE INVENTION

The present application is a continuation-in-part of co-pending U.S. Patent Application Serial No. 60/029,679 filed 30 October 1996, which was a continuation-in-part of U.S. Patent Application Serial No. 60/028,056 filed 02 October 1996, which was a continuation-in-part of U.S. Patent Application Serial No. 60/025,719 filed 10 September 1996. The entire text of each of the above-referenced disclosures is specifically incorporated by reference herein without disclaimer. The government owns rights in the present invention pursuant to grant number DK-20595 and DK-44840 from the National Institutes of Health.

10

### **1. Field of the Invention**

The present invention relates generally to the fields diabetes. More particularly, it concerns the identification of genes responsible for diabetes for use in diagnostics and therapeutics.

15

### **2. Description of Related Art**

Diabetes is a major cause of health difficulties in the United States. Non-insulin-dependent diabetes mellitus (NIDDM also referred to as Type 2 diabetes) is a major public health disorder of glucose homeostasis affecting about 5% of the general population in the United States. The causes of the fasting hyperglycemia and/or glucose intolerance associated with this form of diabetes are not well understood.

20

Clinically, NIDDM is a heterogeneous disorder characterized by chronic hyperglycemia leading to progressive micro- and macrovascular lesions in the cardiovascular, renal and visual systems as well as diabetic neuropathy. For these reasons, the disease may be associated with early morbidity and mortality.

25

Subtypes of the NIDDM can be identified based at least to some degree on the time of onset of the symptoms. The principal type of NIDDM has on-set in mid-life or later. Early-onset NIDDM or maturity-onset diabetes of the young (MODY) shares many features with the more common form(s) of NIDDM whose onset occurs in mid-life. Maturity-onset diabetes of the young (MODY) is a form of non-insulin dependent (Type 2) diabetes mellitus (NIDDM) that is characterized by an early age at onset, usually before 25 years of age, and an autosomal dominant mode of inheritance (Fajans 1989).

Except for these features, the clinical characteristics of patients with MODY are similar to those with the more common late-onset form(s) of NIDDM.

Although most forms of NIDDM do not exhibit simple Mendelian inheritance, the  
5 contribution of heredity to the development of NIDDM has been recognized for many years (Cammidge 1928) and the high degree of concordance of NIDDM in monozygotic twin pairs (Barnett *et al.* 1981) indicates that genetic factors play an important role in its development.

10 MODY is characterized by its early age of onset which is during childhood, adolescence or young adulthood and usually before the age of 25 years. It has a clear mode of inheritance being autosomal dominant. Further characteristics include high penetrance (of the symptomology), and availability of multigenerational pedigrees for 15 genetic studies of NIDDM. MODY occurs worldwide and has been found to be a phenotypically and genetically heterogeneous disorder.

A number of genetically distinct forms of MODY have been identified. Genetic studies have shown tight linkage between MODY and DNA markers on chromosome 20, this being the location of the MODY1 gene (Bell *et al.*, 1991; Cox *et al.*, 1992). MODY2 20 is associated with mutations in the glucokinase gene (GCK) located on chromosome 7 (Froguel *et al.* 1992 and 1993). Recent linkage studies have shown the existence of a further form of MODY which has been termed MODY3 (Vaxillaire *et al.*, 1995). MODY3 has been shown to be linked to chromosome 12 and is localized to a 5 cM 25 region between markers D12S86 and D12S807/D12S820 of the chromosome (Menzel *et al.*, 1995).

Although it is well established that MODY2 is associated with mutations in GCK there is still no information as to the identity of other MODY genes. There is a clear need to identify these genes and the mutations that result in diseased states. The identification 30 of these genes and their products will facilitate a better understanding of the diseased

states associated with mutations in these genes and has important implications in the diagnosis and therapy of MODY.

Since an understanding of the molecular basis of diabetes in general and MODY 5 specifically may facilitate the development of new therapeutic strategies for the treatment of these disorders, studies are needed to identify diabetes-susceptibility genes associated with MODY. Moreover, methods of detecting individuals with a propensity to develop such diseases are needed. Where possible, the molecular mechanism underpinning the genetic lesion should be determined in order to allow diagnosis and specifically-directed 10 therapy

#### SUMMARY OF THE INVENTION

The present invention relates to the inventors discovery that the MODY3 locus the 15 HNF1 $\alpha$  gene, the MODY1 locus is the HNF4 $\alpha$  gene and the MODY4 locus is HNF1 $\beta$ . The invention further relates to the discovery that analysis of mutations in the HNF1 $\alpha$ , HNF1 $\beta$  and HNF4 $\alpha$  genes can be diagnostic for diabetes. The invention also contemplates methods of treating diabetes in view of the fact that mutations in HNF1 $\alpha$ , HNF1 $\beta$  and HNF4 $\alpha$  can cause diabetes.

In one embodiment, the invention contemplates methods for screening for 20 diabetes mellitus. These methods comprise: obtaining sample nucleic acid from an animal; and analyzing the nucleic acids to detect a mutation in an HNF-encoding nucleic segment; wherein a mutation in the HNF-encoding nucleic acid is indicative of a 25 propensity for non-insulin dependent diabetes.

In certain embodiments the HNF-encoding nucleic acid is an HNF1 $\alpha$ -encoding nucleic acid. In view of the inventor's discovery that the MODY3 locus is HNF1 $\alpha$ , a mutation in the HNF1 $\alpha$ -encoding nucleic acid is indicative of a propensity for diabetes.

In some presently preferred embodiments, the HNF1 $\alpha$ -encoding nucleic acid is located on human chromosome 12q, which is the location site of the MODY3 locus. In other embodiments, the HNF-encoding nucleic acid is an HNF4 $\alpha$ -encoding nucleic acid. In view of the inventor's discovery that the MODY1 locus is HNF4 $\alpha$ , a mutation in the 5 HNF4 $\alpha$ -encoding nucleic acid is indicative of a propensity for diabetes. In some presently preferred embodiments, the HNF4 $\alpha$ -encoding nucleic acid is located on human chromosome 20, which is the location of the MODY1 locus.

It is important to note that the terms NIDDM, MODY, MODY1, MODY3, and 10 MODY4 are used to designate diabetes disease states, and the use of a particular such name may not always represent the same causation of that disease state. The inventors have discovered that mutations in HNF4 $\alpha$  can lead to a MODY1 disease state; however, not all mutations in HNF4 $\alpha$  that lead to diabetes might cause a "MODY1" disease state. Conversely, not all diabetes disease states brought about by a mutation in HNF4 $\alpha$  might 15 be considered a MODY1 disease state. Therefore, Applicants prefer to use, in some cases, "HNF4 $\alpha$ -diabetes" to note any diabetic disease state brought on by a mutation or malfunction of HNF4 $\alpha$ , even those that do not exhibit all, or any, MODY1 disease states. Likewise, Applicants may use "HNF4 $\alpha$ -diabetes" and "HNF4 $\beta$ -diabetes" rather than 20 "MODY3" and "MODY4", respectively.

20

The nucleic acid to be analyzed can be either RNA or DNA. The nucleic acid can be analyzed in a whole tissue mount, a homogenate, or, preferably, isolated from tissue to be analyzed. In some preferred embodiments, the step of analyzing the HNF-encoding nucleic acid comprises sequencing of the HNF-encoding nucleic acid to obtain a 25 sequence, the sequence may then be compared to a native nucleic acid sequence of HNF to determine a mutation. Such a native nucleic acid sequence of HNF1 $\alpha$  may have the sequence set forth in SEQ ID NO: 1. Such a native nucleic acid sequence of HNF4 $\alpha$  has a sequence set forth in SEQ ID NO:78.

The method allows for the diagnosis of almost any mutation, including, for example, point mutations, translocation mutations, deletion mutations, and insertion mutations. The method of analysis may comprise PCR, an RNase protection assay, an RFLP procedure, etc. Using this method, the inventors have diagnosed a variety of HNF1 $\alpha$  mutations, including those set forth in Table 8. In preferred embodiments mutations occur at codons 17, 7, 27, 55/56, 98, 131, 122, 142, 129, 131, 159, 171, 229, 241, 272, 288, 289, 291, 292, 273, 379, 401, 443, 447, 459, 487, 515, 519, 547, 548 or 620 of an HNF1 $\alpha$ -encoding nucleic acid nucleic acid, for example, having the sequence of SEQ ID NO:1. In other preferred embodiments a mutation occurs at the splice acceptor region of intron 5 and exon 6 of an HNF1 $\alpha$ -encoding nucleic acid. In other embodiments a mutation occurs at the splice acceptor region of intron 9 of an HNF1 $\alpha$ -encoding nucleic acid. In other embodiments, the mutation occurs independently, in intron 1, intron 2, intron 5, intron 7 or intron 9 of HNF1 $\alpha$  gene. The inventors have also found a variety of HNF4 $\alpha$  mutations, including those found in Table 10. In some preferred embodiments, the HNF-encoding nucleic acid is an HNF4 $\alpha$ -encoding nucleic acid and a mutation occurs in exon 7 of the HNF4 $\alpha$ -encoding nucleic acid. In other preferred embodiments, a mutation occurs at codon 268, 127, 130 or 154 of an HNF4 $\alpha$ -encoding nucleic acid having the sequence of SEQ ID NO:78.

20 The invention also contemplates methods of treating diabetes in an animal comprising: diagnosing an animal that has diabetes and modulating HNF function in the animal.

25 The step of diagnosing an animal with diabetes frequently comprises analysis of an HNF1 $\alpha$ -encoding nucleic acid sequence or an HNF4 $\alpha$ -encoding nucleic acid sequence for a mutation.

The step of modulating HNF function may comprise providing an HNF1 $\alpha$  or HNF4 $\alpha$  polypeptide to the animal. In cases where normal HNF1 $\alpha$  or HNF4 $\alpha$  function is

sought to be revived, the HNF1 $\alpha$  or HNF4 $\alpha$  polypeptide may be a native HNF1 $\alpha$  or HNF4 $\alpha$  polypeptide. For example, a native HNF1 $\alpha$  polypeptide may have the sequence of SEQ ID NO: 2. A native HNF4 $\alpha$  polypeptide may have the sequence of SEQ ID NO: 79. The provision of an HNF1 $\alpha$  or HNF4 $\alpha$  polypeptide is accomplished by any of a number of ways. For example, expression of an HNF1 $\alpha$  or HNF4 $\alpha$  polypeptide may be induced, with the expression being of an HNF1 $\alpha$  or HNF4 $\alpha$  polypeptide encoded in the animal's genome or of an HNF1 $\alpha$  or HNF4 $\alpha$  polypeptide encoded by a nucleic acid provided to the animal. The provision of an HNF1 $\alpha$  or HNF4 $\alpha$  polypeptide may be accomplished by a method comprising introduction of an HNF1 $\alpha$  or HNF4 $\alpha$ -encoding nucleic acid to the animal, for example, by injecting the HNF1 $\alpha$  or HNF4 $\alpha$ -encoding nucleic acid into the animal.

Modulating HNF function in the animal can comprise providing a modulator of HNF1 $\alpha$  or HNF4 $\alpha$  function to the animal. Such modulators are in the nature of drugs and can be, for example HNF4, HNF6, HNF3 or any other peptide or molecule that regulates HNF1 $\alpha$ . These modulators may be formulated into a pharmaceutical compound for delivery to the animal. The modulator of HNF1 $\alpha$ , HNF $\beta$  or HNF4 $\alpha$  function may be an agonist or antagonist of HNF1 $\alpha$ , HNF $\beta$  or HNF4 $\alpha$ . The modulator may modulate transcription of an HNF1 $\alpha$ , HNF $\beta$  or HNF4 $\alpha$ -encoding nucleic acid, translation of an HNF1 $\alpha$ , HNF $\beta$  or HNF4 $\alpha$ -encoding nucleic acid, or the functioning of the HNF1 $\alpha$ , HNF $\beta$  or HNF4 $\alpha$  polypeptide.

The invention also contemplates methods of screening for modulators of HNF function comprising: obtaining an HNF polypeptide, for example an HNF1 $\alpha$ , HNF $\beta$  or HNF4 $\alpha$  polypeptide; determining a standard activity of the HNF; contacting the polypeptide with a putative modulator; and assaying for a change in the standard activity of the polypeptide. In some preferred methods, the standard activity profile of a HNF1 $\alpha$  polypeptide is determined by measuring the binding of the HNF1 $\alpha$  polypeptide to a

nucleic acid segment comprising the sequence of SEQ ID NO: 9. To facilitate measuring the HNF1 $\alpha$  activity, the nucleic acid segment comprising the sequence of SEQ ID NO: 9 or the HNF1 $\alpha$  polypeptide may comprise a detectable label. In some preferred methods, the standard activity profile of a HNF4 $\alpha$  polypeptide is determined by measuring the  
5 binding of the HNF4 $\alpha$  polypeptide to a nucleic acid segment comprising the sequence of SEQ ID NO: 85. To facilitate measuring the HNF4 $\alpha$  activity, the nucleic acid segment comprising the sequence of SEQ ID NO: 85 or the HNF4 $\alpha$  polypeptide may comprise a detectable label. In other embodiments, the standard activity profile of an HNF polypeptide is determined by determining the ability of an HNF1 $\alpha$  polypeptide to  
10 stimulate transcription of a reporter gene, the reporter gene operatively positioned under control of a nucleic acid segment comprising the sequence of SEQ ID NO: 1. In other embodiments, the standard activity profile of an HNF polypeptide is determined by determining the ability of an HNF4 $\alpha$  polypeptide to stimulate transcription of a reporter gene, the reporter gene operatively positioned under control of a nucleic acid segment  
15 comprising the sequence of SEQ ID NO: 78. Similar assays are contemplated for HNF1 $\beta$  polypeptide.

The invention also contemplates methods of screening for modulators of HNF polypeptide function comprising: obtaining an HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$ -encoding  
20 nucleic acid segment; determining a standard transcription and translation activity of the HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$ -encoding nucleic acid sequence; contacting the HNF1 $\alpha$  or HNF4 $\alpha$ -encoding nucleic acid segment with a putative modulator; maintaining the nucleic acid segment and putative modulator under conditions that normally allow for  
25 HNF1 $\alpha$  or HNF4 $\alpha$  transcription and translation; and assaying for a change in the transcription and translation activity.

The inventors discovery allows for the preparation of a host of HNF modulators such as MODY3/HNF1 $\alpha$ -modulators, MODY4/HNF1 $\beta$ -modulators and MODY1/HNF4 $\alpha$  modulators. Such modulators themselves are within the scope of the

invention. Such an HNF modulator may be prepared or preparable by a process comprising screening for modulators of HNF function comprising: obtaining an HNF polypeptide; determining a standard activity profile of the HNF polypeptide; contacting the HNF polypeptide with a putative modulator; and assaying for a change in the standard 5 activity profile. An HNF modulator prepared by a process comprising screening for modulators of HNF function comprising: obtaining an HNF-encoding nucleic acid segment; determining a standard transcription and translation activity of the HNF-nucleic acid sequence; contacting the HNF-encoding nucleic acid segment with a putative modulator; maintaining the nucleic acid segment and putative modulator under conditions 10 that normally allow for HNF transcription and translation; and assaying for a change in the transcription and translation activity.

Some aspects of the invention relate to isolated and purified polynucleotides encoding an HNF polypeptide. Such polynucleotides can be: an HNF1 $\alpha$ -encoding 15 nucleic acid, HNF1 $\beta$ -encoding nucleic acid sequence, or an HNF4 $\alpha$ -encoding nucleic acid. In some particular embodiments, the polynucleotide encodes an HNF1 $\alpha$  having an amino acid sequence as set forth in SEQ ID NO:127. In preferred embodiments, the polynucleotide may be an HNF1 $\alpha$ -encoding nucleic acid sequence has a sequence of SEQ 20 ID NO:126. In additional particular embodiments, the polynucleotide encodes an HNF1 $\beta$  having an amino acid sequence as set forth in SEQ ID NO:139. In preferred embodiments, the polynucleotide may be an HNF1 $\beta$ -encoding nucleic acid sequence having a sequence of SEQ ID NO:128. The polynucleotide may encode an HNF4 $\alpha$  having an amino acid sequence as set forth in SEQ ID NO:140. In preferred 25 embodiments, the polynucleotide may be an HNF4 $\alpha$ -encoding nucleic acid sequence has a sequence of SEQ ID NO:130.

Other embodiments comprise isolated and purified nucleic acid segments comprising 10, 14, 15, 25, 30, 35, 40, 45, 50, 55, 60, 70, 80, 90, 100, 125, 150, 175, 200, 250, 300, 350, 400, 450, or 500 contiguous nucleic acids identical to the sequence of SEQ 30 ID NO:128 or SEQ ID NO: 126 or the complement of these sequences. These nucleic

acid segments can be used by those of skill in the art as hybridization probes, PCR primers, for the expression of HNF polypeptides, for the expression of other polypeptides, etc. In some embodiments, the segment encodes a full-length HNF polypeptide. Of particular interest are the promoters for HNF1 $\alpha$  and HNF1 $\beta$ , which are disclosed in SEQ ID NOS: 126 and 128 respectively and in FIGs. 26 and 27, respectively and discussed elsewhere in this application. These promoters may be used by those of skill in the art in many varying applications.

#### BRIEF DESCRIPTION OF THE DRAWINGS

10

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

15

**FIG. 1.** Pedigrees of MODY3 families. The individuals studied in the Clinical Research Center at the University of Chicago are indicated by MD-1-5 and 8-13 and those with NIDDM, IGT and NGT are shown by black symbols, shaded symbols and open symbols, respectively. The asterisks indicate that these individuals have inherited the at-risk haplotype associated with MODY3 in that family. The genotypes and haplotypes for the P family have been described (Menzel *et al.*, 1995) and the pairwise lod score between MODY and the D12S76/D12S321 haplotype in this family is 2.06 at a recombination fraction of 0.00. The pairwise lod score between MODY and D12S76 in pedigree F549 is 0.65 at a recombination fraction of 0.00 (Vaxillaire *et al.*, 1995). The pedigrees BDA1 and BDA12 have not been previously described. MODY co-segregates with markers tightly linked to MODY3 in these families with pairwise lod scores between MODY and D12S86 of 1.94 and 0.60, respectively, at a recombination fraction of 0.00.

20

25

**FIG. 2.** Average glucose (A), insulin (B) and insulin secretion rate (ISR) (C) profiles in 7 diabetic MODY3 subjects ( $\square$ ), 6 nondiabetic MODY3 subjects ( $\blacktriangle$ ) and 6

30

control subjects (o), during the stepped glucose infusion studies. After a 30 min period of baseline sampling, glucose was infused at rates of 1, 2, 3, 4, 6, and 8 mg  $\cdot$ kg $^{-1}$   $\cdot$ min $^{-1}$ . Each infusion rate was administered for a period of 40 min and glucose, insulin and C-peptide were measured at 10, 20, 30 and 40 min into each period.

5

**FIG. 3.** Relationship between average plasma glucose concentrations and ISR's during the stepped glucose infusion studies in 7 diabetic MODY3 subjects ( $\square$ ), 6 nondiabetic MODY3 subjects ( $\blacktriangle$ ) and 6 control subjects (o). The lowest glucose levels and ISR's were measured under basal conditions, and subsequent levels were obtained during glucose infusion rates of 1, 2, 3, 4, 6 and 8 mg kg $^{-1}$   $\cdot$ min $^{-1}$ , respectively.

10

**FIG. 4.** Graded intravenous glucose infusions were administered to 6 controls (A), 6 nondiabetic MODY3 subjects (B) and 7 diabetic MODY3 subjects (C) after an overnight fast (baseline ( $\blacktriangle$ )) and after a 42-h intravenous infusion of glucose (postglucose ( $\square$ )) at a rate of 4-6 mg kg $^{-1}$   $\cdot$ min $^{-1}$ .

15

**FIG. 5A, FIG. 5B, FIG. 5C, FIG. 5D, FIG. 5E, FIG. 5F and FIG 5G.** MODY3 pedigrees showing co-segregation of mutant HNF1 $\alpha$  allele with diabetes mellitus. Males are noted by square symbols and females by circles. Individuals with NIDDM are noted by black symbols and those with gestational-onset diabetes or impaired glucose tolerance by shaded symbols. A diagonal line through the symbol indicates that the individual is deceased.

20

The individual ID is noted at the top right corner of each symbol and the HNF1 $\alpha$  genotype, if determined, noted below: N, normal allele; M, mutant allele. The arrow indicates the individual from each pedigree who was screened for mutations. Note that some individuals have inherited the mutant allele but do not yet have NIDDM, usually because of their young age (e.g. P pedigree, individual IV-6; and Ber pedigree, individual V-2. Also, some individuals have NIDDM even though they did not inherit the mutant HNF1 $\alpha$  allele segregating in that family (e.g. Ber pedigree, individual II-2). Such

30

heterogeneity has been noted previously (Bell *et al*, 1991) and is a reflection of the high prevalence of NIDDM.

FIG. 6. The involvement of hepatocyte nuclear factors in diabetes.

5

FIG. 7. An alignment of the HNF4 $\alpha$  protein sequence from humans (h) with sequences from human, mouse (m), Xenopus (x) and Drosophila (d) species. The putative DNA binding sites are underlined and the putative ligand binding sites are in bold.

10

FIG. 8A, FIG. 8B, FIG. 8C, FIG. 8D FIG. 8E, FIG. 8F, FIG. 8G, FIG. 8I, FIG. 8H, FIG. 8I. The DNA sequences for exon 1, exon 2, exon 3, exon 4, exon 5 exon 6 exon 7 exon 8 exon 9 and exon 10 of HNF4 $\alpha$ .

15

FIG. 9. Physical map of the MODY3 region of chromosome 12. YAC, BAC (b) and PAC (p) clones are represented as lines, the length of which reflects the number of included STSs and not the actual size. The physical distance between adjacent STSs has not been determined directly and STSs for which the order has not been unambiguously determined are indicated in brackets. A circle indicates that the clone was positive for the indicated STS and a square indicates a STS derived from the end of that specific clone. Several YACs contain large internal deletions which are noted by brackets. The STSs are from GDB<sup>TM</sup> and the GenBank STS databases.

20

FIG. 10. Partial sequence of exon 4 of the HNF-1 $\alpha$  gene of individual EA1 (Edinburgh pedigree). The sequences of the normal and mutant alleles are shown. There is an insertion of a C in codon 291 (noted by the arrowhead) in the mutant allele resulting in a frameshift and premature termination.

25

FIG. 11. The cDNA sequence of HNF1 $\alpha$  denoting position of the exons.

**FIG. 12.** Model of the human HNF-4 $\alpha$  showing the different patterns of alternative splicing and structures of the different forms of HNF-4 $\alpha$  that can be generated by alternative splicing. The amino acids that define the boundaries of some of the regions of the protein are shown. DBD and LBD correspond to the DNA and ligand-binding domains of HNF-4 $\alpha$ , respectively.

**FIG. 13.** Comparison of the sequences of the promoter regions of the human and mouse HNF-4 $\alpha$  genes (SEQ ID NO:135 and SEQ ID NO:137, respectively). Identical residues are shown in boxes. The binding sites for transcription factors that may regulate the expression of HNF-4 $\alpha$  are overlined. The asterisk notes the predicted transcriptional start site based on the study of the mouse HNF-4 $\alpha$  gene (Zhong *et al.*, 1994). The minimal promoter region required for high-level expression of the mouse gene in hepatoma cells is shown by shading. The ATG codon which defines the start of translation is noted. The arrowhead shows the DNA polymorphism found in the promoter region of the proband of family J2-96. The GenBank accession nos. for the mouse promoter sequence are S74519 and S77762.

**FIG. 14A and FIG. 14B.** Partial sequence of exon 4 of HNF4 $\alpha$  gene of patient J2-21. The sequences of the normal (FIG. 14A SEQ ID NO:141 and corresponding amino acids SEQ ID NO:142) and mutant (FIG. 14B; SEQ ID NO:143) alleles are shown and the arrow indicates the C $\rightarrow$ T substitution at codon 127.

**FIG. 15.** Pedigrees of Japanese families with mutations/polymorphisms in the HNF-4 $\alpha$  gene. Individuals with diabetes are noted by filled symbols and nondiabetic (or not tested) individuals are indicated by open symbols. The arrow indicates the proband. The clinical features of each subject are shown including age at diagnosis, present age and present treatment. The HNF4 $\alpha$  genotype of tested individuals is noted: N-normal and M-mutation/polymorphism.

FIG. 16. Identification of a nonsense mutation in the HNF4 $\alpha$  gene in a german family, the Dresden-11 pedigree. The members of this family with MODY and impaired glucose tolerance are indicated with black and shaded symbols, respectively. The age at diagnosis of diabetes mellitus, present age and therapy (OHA, oral hypoglycemic agents), and nature of complications (M, macrovascular disease; R, retinopathy; and N, peripheral polyneuropathy) are indicated. The haplotype associated with MODY in this family is shown.

FIG. 17. Partial sequence of exon 4 of the HNF4 $\alpha$  gene of subject II-4 of the Dresden-11 pedigree. The R154X mutation is indicated (SEQ ID NO:144 and SEQ ID NO:145). Intron 4 follows the Gln codon, CAG.

FIG. 18A, FIG. 18B, FIG. 18C and FIG. 18D. Oral glucose tolerance testing in the Dresden-11 family. The blood glucose (FIG. 18A), insulin (FIG. 18B), C-peptide (FIG. 18C) and proinsulin (FIG. 18D) levels during the course of the glucose tolerance test are shown. The open symbols are the means $\pm$ SEM for subjects with the R154X mutation, including those with diabetes and impaired glucose tolerance, and the filled symbols are the means for the two normal subjects.

FIG. 19A, FIG. 19B, FIG. 19C and FIG. 19D. Effect of bolus and infusion of arginine, of glucose, and of arginine during hyperglycemic clamp on plasma concentration of glucose (FIG. 19A), insulin (FIG. 19B), C-peptide (FIG. 19C), and glucagon (FIG. 19D) in 3 groups of subjects of the RW pedigree.

FIG. 20A and FIG. 20B. Acute insulin (FIG. 20A) and C-peptide (FIG. 20B) response to bolus administration of arginine in 3 groups of subjects of the RW pedigree at baseline and during the hyperglycemic clamp procedure. The slope of the line connecting these insulin responses (slope of potentiation) was lower in ND[+] vs. ND[-], p < 0.001. The slope for D[+] was lowest.

30

**FIG. 21.** MODY pedigree, Italy-1. Subjects with MODY and impaired glucose tolerance are indicated by filled and cross-hatched symbols, respectively. Nondiabetic subjects (by testing or history) are indicated by open symbols. The clinical features of the subjects are noted below the symbol including current treatment: insulin or oral hypoglycemic agent (OHA). The haplotype at the markers D12S321-D12S76-UC-39 is shown and the at-risk haplotype is noted by shading. The HNF-1 $\alpha$  genotype is shown: N, normal; M, mutant (A $\rightarrow$ C substitution at nucleotide -58). Although treated insulin, subject III-9 fasting C-peptide value of 1.2 ng/ml indicating that she has MODY rather than insulin-dependent diabetes mellitus.

10

**FIG. 22.** Comparison of the sequence of the promoter region of the human, rat, mouse, chicken and frog HNF-1 $\alpha$  genes (SEQ ID NO:134; SEQ ID NO:138; SEQ ID NO:136; SEQ ID NO:132; SEQ ID NO:133 respectively). The A $\rightarrow$ C substitution at nucleotide -58 and HNF-4 $\alpha$  binding site are shown. Residues identical to the human sequence are boxed. Nucleotides are numbered relative to the transcriptional start site of the human gene (indicated by an asterisk). The boxed ATG triplet is the initiating methionine. The dashes indicate gaps introduced in the sequences to generate this alignment.

20           **FIG. 23.** Summary of mutations in the human HNF-1 $\alpha$  gene. This cartoon shows the exons and promoter region as boxes. The mutations and amino acid polymorphisms are from Yamagata *et al.*, 1996; Lehto M, *et al.*, 1997; Kaisaki PJ, *et al.*, 1997; Vaxillaire *et al.*, 1997; Frayling *et al.*, 1997; Hansen T, *et al.*, 1997; Urhammer *et al.*, 1997; Glucksmann *et al.*, 1997. The amino acid polymorphisms are I/L27, A/V98 and S/N487. The single-letter abbreviations for the amino acids are used.

25

**FIG. 24** Partial sequence of exon 2 of HNF-1 $\beta$  gene of subject J2-20 (SEQ ID NO:146 and SEQ ID NO:147). The C $\rightarrow$ T mutation in codon 177 is indicated.

**FIG. 25.** J2-20 pedigree. Individuals with diabetes mellitus are noted by filled symbols. The arrow indicates the proband. The present age, age at diagnosis, current treatment and complications are shown. The HNF-1 $\beta$  genotype is noted: N, normal; M, mutant. OHA, oral hypoglycemic agent; PDR, proliferative diabetic retinopathy; CRF, 5 chronic renal failure; and DKA, diabetic ketoacidosis.

**FIG. 26A-FIG. 26M** Partial sequence of human HNF1 $\alpha$  gene. These figures depict a contiguous sequence and have been split into panels due to the size of the sequence. The nucleotide and predicted amino acid sequences are shown. Exon and 10 intron sequences are in uppercase and lower cases respectively. The approximate size of the gaps in the introns, the complete sequence of which was not determined are noted. In the promoter region, potential binding sites for transcription factors that may regulate expression of this gene are indicated, with sites identified by Dnase footprinting in italics, those identified by sequence homology in normal type. The minimal promoters region is 15 shown in boldface type. The polymorphisms and mutations in the HNF1 $\alpha$  gene identified to date are shown in boldface type with the designation of the mutation noted. The asterisk notes the predicted transcriptional start site based on studies of rat HNF1 $\alpha$  gene. The letter n indicates that the sequence was ambiguous at this site

**FIG. 27A-FIG. 27I** Partial sequence of human HNF1 $\beta$  gene. These figures depict a contiguous sequence and have been split into panels due to the size of the sequence. The nucleotide and predicted amino acid sequences are shown. Exon and intron sequences are in uppercase and lower cases respectively. The approximate size of the gaps in the introns, the complete sequence of which was not determined are noted. In 20 the promoter region, potential binding sites for transcription factors that may regulate expression of this gene are indicated, with sites identified by Dnase footprinting in italics, those identified by sequence homology in normal type.

**FIG. 28A-FIG. 28V** Partial sequence of human HNF4 $\alpha$  gene. These depict a 30 contiguous sequence and have been split into panels due to the size of the sequence. The

nucleotide and predicted amino acid sequences are shown. Exon and intron sequences are in uppercase and lower cases respectively.

#### **DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS**

5

The present invention concerns the early detection, diagnosis, prognosis and treatment of diabetes. The present invention describes for the first time mutations responsible for HNF1 $\alpha$ , HNF1 $\beta$  and HNF4 $\alpha$ -related diabetes. The specific mutation and identity of the corresponding wild-type genes from diabetic subjects, are disclosed. These 10 mutations are indicators of HNF1 $\alpha$ , HNF1 $\beta$  and HNF4 $\alpha$  related diabetes and are diagnostic of the potential for the development of diabetes. It is envisioned that the techniques disclosed herein will also be used to identify other gene mutations responsible for other forms of diabetes.

15

Those skilled in the art will realize that the nucleic acid sequences disclosed will find utility in a variety of applications in diabetes detection, diagnosis, prognosis and treatment. Examples of such applications within the scope of the present invention include amplification of markers of MODY using specific primers; detection of markers of HNF1 $\alpha$ , HNF1 $\beta$  and HNF4 $\alpha$  by hybridization with oligonucleotide probes; incorporation of isolated 20 nucleic acids into vectors and expression of vector-incorporated nucleic acids as RNA and protein; development of immunologic reagents corresponding to gene encoded products; and therapeutic treatment for the identified MODY using these reagents as well as, anti-sense nucleic acids, or other inhibitors specific for the identified MODY. The present invention further discloses screening assays for compounds to upregulate gene expression or 25 to combat the effects of the mutant HNF1 $\alpha$ , HNF1 $\beta$  and HNF4 $\alpha$  genes.

#### **A. DIABETES AND MODY**

Diabetes mellitus affects approximately 5% of the population of the United States 30 and over 100 million people worldwide (King *et al.*, 1988, Harris *et al.*, 1992). A better way of identifying the populace who are at risk of developing diabetes is needed as a

subject may have normal plasma glucose compositions but may be at risk of developing overt diabetes. These issues could be resolved if it were possible to diagnose susceptible people before the onset of overt diabetes. This is presently not possible with subjects having classical diabetes due to its multifactorial nature.

5

MODY is a monogenic form of diabetes and thus the genes responsible can be more easily studied than those whose mutation contributes to the development of polygenic form(s) of this disorder such as type 1 and type 2 diabetes mellitus. Recent studies have shown that subjects with maturity onset diabetes of the young (MODY), a 10 subset of diabetes characterized by diabetes in the first or second decade of life and autosomal dominant inheritance have shown that MODY may result from mutations in genes on chromosome 20 (HNF4 $\alpha$ /MODY1), chromosome 7 (glucokinase/MODY2) chromosome 12 (HNF1 $\alpha$ /MODY3) and chromosoem 17 (HNF1 $\beta$ /MODY4).

15

The clinical characteristics that manifest in HNF4 $\alpha$ , HNF1 $\alpha$  and HNF1 $\beta$  type diabetes resemble those seen in patients with type 2 diabetes. These characteristics include frequent severe fasting hyperglycemia, the need for oral hypoglycemic agents, eventual insulin requirements, and vascular and neuropathic complications (Fajans *et al.*, 1994; Menzel *et al.*, 1995).

20

The inventors have shown that prediabetic subjects with mutations in the HNF1 $\alpha$  and HNF4 $\alpha$  genes have subtle but important alterations in the normal pattern of glucose-stimulated insulin secretion. Compared to control subjects with no family history of diabetes, they had normal insulin secretion rates at lower glucose concentrations. 25 However the increase in insulin secretion rate resulting from an increase in the plasma glucose concentration above 8 mM was less in prediabetic HNF1 $\alpha$ -mutation subjects than controls (see FIG. 2- FIG. 4).

Exposure of the normal  $\beta$ -cell to increased plasma glucose concentrations for 42-30 hours results in an increase in  $\beta$ -cell responsiveness to a subsequent glucose stimulus.

Following a 42-hr glucose infusion which raised the plasma glucose concentration to an average value of  $7.1 \pm 1.4$  mM, the insulin secretion rate of prediabetic HNF1 $\alpha$ -mutation subjects increased by 35% between 5-9 mM glucose with a resultant shift in the dose-response curve to the left. Five out of six prediabetic HNF1 $\alpha$ -mutation subjects showed  
5 this increase in insulin secretion rate, and only one subject MD13 failed to demonstrate this effect. The magnitude of this priming effect of glucose was similar to that seen in the controls.

Diabetic HNF1 $\alpha$ -mutation subjects demonstrated diminished insulin secretion  
10 across the entire range of glucose concentrations studied. Thus, over the concentration range between 5 and 9 mM glucose, the diabetic subjects secreted 50% less insulin than the controls and 51% less than the prediabetic HNF1 $\alpha$ -mutation subjects. Furthermore, the priming effect of glucose was lost in the subjects with overt diabetes.

15 Evaluation of insulin resistance indicated that HNF1 $\alpha$ -mutation subjects were no more resistant than the controls. In fact, there was a tendency towards a lesser degree of insulin resistance in the HNF1 $\alpha$ -mutation subjects, making it highly unlikely that insulin resistance plays a primary role in the pathophysiology of diabetes in these subjects.

20 The inventors have recently characterized insulin secretory responses in prediabetic HNF4 $\alpha$  and HNF1 $\alpha$ -mutation subjects. Prediabetic HNF4 $\alpha$  and HNF1 $\alpha$ -mutation subjects both have reduced insulin secretory responses to glucose which are evident only as the plasma glucose rises above a threshold of 7 or 8 mM, respectively. Whereas in HNF1 $\alpha$ -mutation subjects the priming effect of glucose on insulin secretion  
25 is retained, a low-dose glucose infusion did not have any significant effects on insulin secretion in prediabetic HNF4 $\alpha$ -mutation subjects (Byrne *et al.*, 1995b). In subjects with mutations in the glucokinase gene, the dose-response curve is shifted to the right and ISR is markedly decreased at glucose concentrations below 7 mM, but insulin secretion continues to increase with increasing plasma glucose concentrations even above levels of

8 mM. The priming effect of glucose on insulin secretion also is preserved (Byrne *et al.*, 1994). The inventors have recently performed similar studies in subjects with classical Type 2 and impaired glucose tolerance. In subjects with IGT, although the dose-response curve relating glucose and insulin secretion was shifted to the right, the priming effect of 5 glucose on insulin secretion was retained. In subjects with overt Type 2 diabetes, the increase in insulin secretion in response to an increase in glucose was markedly reduced and the priming effect of glucose on insulin secretion was lost.

It thus appears that  $\beta$ -cell dysfunction plays an important, pathophysiologic role in 10 the development of the three forms of MODY which have been characterized to date. A clear prediabetic phase has not been identified in subjects with glucokinase mutations. However, profound defects in the ability of the  $\beta$ -cell to respond to a glucose stimulus is present even in the face of the mild elevations in glucose which characterizes the majority 15 of these subjects. By contrast, a prediabetic phase is a feature of the HNF4 $\alpha$  and HNF1 $\alpha$  forms of diabetes. These prediabetic subjects have reduced insulin secretory responses to elevated concentrations of glucose induced by the step-wise glucose infusion prior to onset of diabetes. Prediabetic HNF4 $\alpha$  and HNF1 $\alpha$  subjects can be distinguished based 20 on the effects of a low dose glucose infusion on insulin secretion. The priming effect of glucose on insulin secretion is retained in HNF1 $\alpha$  subjects in the prediabetic phase but is lost after the onset of overt hyperglycemia whereas this priming effect is absent in HNF4 $\alpha$  diabetes even in the prediabetic phase of the disease. The severe reductions in 25 insulin secretory responses to glucose seen in the overtly diabetic HNF1 $\alpha$  subjects are likely to be due in part to the effects of high glucose, in view of the well documented adverse effects of hyperglycemia on insulin secretion. A full understanding of the reasons for these changes in the dose-response relationships between glucose and insulin 30 secretion requires a better understanding of the roles of HNF4 $\alpha$  and HNF1 $\alpha$  in regulating normal pancreatic  $\beta$ -cell function.

Further studies by the inventors have shown that elevations in the 2-hr post- 30 challenge blood glucose levels predict alterations in insulin secretory responses to

glucose. However, in that case, subjects with impaired glucose tolerance demonstrated reduced insulin secretory responses over a range of glucose concentrations and not just in response to increases in glucose above 8 mM as was seen in the prediabetic HNF1 $\alpha$ -mutation subjects. Thus, the inventors do not believe that the alterations in insulin secretion seen in the prediabetic HNF1 $\alpha$  subjects resulted from the modest elevations in glucose. Rather, the inventors' results suggest that the percent priming and overall insulin secretion rates deteriorate as glucose tolerance deteriorates, and the lack of ability to increase insulin secretion at high glucose levels is a feature of the mutation in the HNF1 $\alpha$  gene.

10

From the studies described above and in the Examples that follow it is clear that the identification and characterization of the gene(s) associated with MODY diabetes is important. Mutations in such genes lead to diabetes and it would be diagnostically and therapeutically advantageous to identify the mutations in subjects predisposed to such mutations.

15

Studies attempting to find the location of the MODY3 gene showed that the putative gene linked to MODY3 type diabetes was localized to a 5 cM interval between the markers D12S86 and D12S807/D12S820 (Menzel *et al.*, 1995). However the identity 20 of the gene has not been elucidated. The present invention for the first time shows that the gene linked to MODY3 expresses a factor previously identified from hepatocyte known as hepatocyte nuclear factor 1  $\alpha$  herein referred to as HNF1 $\alpha$ .

25

Similarly studies attempting to find the location of the MODY1 gene showed that the putative gene linked to MODY1 type diabetes was localized to a 13 cM interval between the markers D20S169 and D20S176 (Stoffel *et al.*, 1996). Likewise, as with MODY3, the identity of the gene in MODY1 has not been elucidated. The present invention for the first time shows that the gene linked to MODY1 expresses a factor previously identified from hepatocytes known as hepatocyte nuclear factor 4  $\alpha$  herein 30 referred to as HNF4 $\alpha$ .

Subsequently, the inventors performed studies to elucidate the genetic defects responsible for other forms of MODY. The present invention for the first time shows that MODY is likely a consequence of mutations in hepatocyte nuclear factor 1 $\beta$  herein referred to as HNF1 $\beta$ .

The association of mutation in HNF1 $\alpha$ , HNF1 $\beta$  and HNF4 $\alpha$  with diabetes indicates the importance of the HNF network in controlling pancreatic  $\beta$ -cell function and glucose homeostasis. Hence the studies presented here have categorized exemplary mutations in the HNF1 $\alpha$ , HNF1 $\beta$  and HNF4 $\alpha$  genes as identified by PCR techniques. These landmark results form the basis of many therapeutic and diagnostic techniques as measures to alleviate diabetes, particularly HNF 1 $\alpha$ -diabetes, HNF 1 $\beta$ -diabetes and HNF 4 $\alpha$ -diabetes.

15      **B.     HEPATOCYTE NUCLEAR FACTORS ARE THE GENES LINKED TO MODY TYPE DIABETES.**

*Hepatocyte Nuclear Factor 1 $\alpha$*

20       Hepatic nuclear factor 1 $\alpha$  (also known as APF, LFB1 or HP1) has been described as a sequence specific DNA binding protein from rat liver. It is thought to interact with promoter elements present in many genes including albumin,  $\alpha$ - and  $\beta$ - fibrinogen,  $\alpha$ -1-antitrypsin,  $\alpha$ -fetoprotein pyruvate kinase, transthyretin and aldose B among others. HNF1 $\alpha$  has been purified from rat liver extracts by DNA affinity chromatography using 25 fibrinogen promoter element (Courtoise, 1987) and was characterized as a single 88 kDa protein. It is now known that HNF1 $\alpha$  is a transcription factor.

30       Mendel and Crabtree (1993) suggested that HNF1 $\alpha$  interacted with "hepatocyte-specific" genes in which it plays a prominent role in regulation of both *in vitro* and *in vivo* transcription. However, it was later shown that HNF1 $\alpha$  mRNA can also be found in

several non-hepatocyte tissues including the kidney, stomach, intestines, thymus and spleen and pancreas (Baumhueter *et al.*, 1990; Kuo *et al.*, 1990). This suggests that HNF1 $\alpha$  expression may participate in the differentiation of non-hepatic organs as well as hepatogenesis.

5

Transcription factors are proteins that control transcription by binding to cis-acting regulatory DNA sequences in a gene. As such, these factors play a crucial role in development and differentiation by dictating the pattern of expression of genes within specific cells and tissues.

10

The homeodomain proteins are a class of transcription factors. These proteins all possess the unusual characteristic of having very similar DNA-binding domains even though they mediate diverse effects. HNF1 $\alpha$  is an example of a homeodomain protein. HNF1 $\alpha$  has been shown to dimerize with itself in solution. It appears that maximal 15 transcriptional activation by HNF1 $\alpha$  requires a novel dimerization cofactor. This cofactor, known as the dimerization cofactor of HNF1 $\alpha$  (DCoH), does not in itself bind DNA, rather, it binds HNF1 $\alpha$ .

HNF1 $\alpha$  binds to DNA as a dimer; this was confirmed from studies on the 20 purification and cloning of HNF1 $\alpha$ . Other studies showed that there was a DNA binding protein that binds to the HNF1 $\alpha$  binding site in cells that lack the HNF1 $\alpha$  mRNA. This second protein HNF1 $\beta$  is a homolog of HNF1 $\alpha$  but is the product of a separate gene.

Regulation studies of the HNF1 $\alpha$  promoter showed that binding sites for 25 transcription factors HNF3, AP1 and HNF4 $\alpha$  are essential for the expression of HNF1 $\alpha$  (Hansen and Crabtree, 1993). It has been demonstrated that HNF4 $\alpha$  is located on chromosome 20 of the human genome. The present inventors suggest that MODY1, which is known to be linked to chromosome 20, may act as a regulator of MODY3 gene

expression as such mutations in HNF4 $\alpha$  may be responsible for MODY1 form of diabetes.

HNF1 $\alpha$  proteins possess three functional regions, namely, the dimerization, 5 activation and DNA-binding domains. The dimerization domain is localized to the first 32 amino acids of the HNF1 $\alpha$  proteins. The DNA-binding domain is a POU-like homeodomain which binds to a 13 bp palindromic DNA sequence in the promoters of HNF1 $\alpha$  binding proteins (Courtois *et al.*, 1988; Frain *et al.*, 1989). The consensus sequence for this HNF1 $\alpha$  binding site on these genes is:

10

GTAAATNATTACC (SEQ ID NO:9)

Diabetes mellitus alters the transcription of numerous genes in many different tissues. The mechanisms underlying these alterations in transcription are largely 15 unknown. One example of altered transcription is seen in the reduced transcription of the albumin gene in diabetes (Wanke *et al.*, 1991). Recently, it has been demonstrated that HNF1 $\alpha$  protein levels are reduced in diabetes, leading to the theory that decreased gene transcription in diabetes is due to decreased levels of HNF1 $\alpha$  a factor critical for the regulation of hepatic albumin gene expression. This is thought to be the case in other 20 genes that posses an HNF1 $\alpha$  binding site and are affected by diabetes. Therefore changes in the abundance of HNF1 $\alpha$  in diabetes appears to affect the expression of genes whose expression is predominantly regulated by this factor.

The expression of the insulin gene in adult mammals is localized to the  $\beta$  cells in 25 the pancreatic islets. Studies of this gene have defined a small region in the promoter, the FF-minienhancer, capable of conferring tissue-specific and glucose responsive transcriptional activity on a heterologous promoter (German *et al.*, 1990). This minienhancer region is composed of two primary regulatory elements the Far box and the FLAT element which interact to upregulate transcription.

Further analysis of the FLAT element showed it to be a cluster of several cis loci that mediate discrete positive and negative effects. The positive locus is characterized as FLAT-F and its activity is only revealed when there is a mutation in the negative locus  
5 FLAT-E. This FLAT-F region is able to specifically bind a number of DNA-binding proteins. The sequence of FLAT-F has significant similarity to the consensus sequence of HNF1 $\alpha$ . This led to studies to determine whether HNF1 $\alpha$  itself may play a role in the transcriptional regulation of the rat insulin gene. Subsequently, it was shown that HNF1 $\alpha$  expression is present in the pancreatic  $\beta$ -cell derived insulinoma cell line HIT. HNF1 $\alpha$   
10 has been shown to bind with and transactivate rat insulin gene enhancers that contain an HNF1 $\alpha$  site.

#### *Hepatocyte Nuclear Factor 4 $\alpha$*

15 Hepatocyte nuclear factor 4 $\alpha$  (HNF4 $\alpha$ ) is another transcription factor first associated with the liver and having limited tissue distribution (Xanthopoulos *et al.*, 1991; Zhong *et al.*, 1994). HNF4 $\alpha$  can activate transcription in several non-hepatic cell lines, indicating that no liver-specific modification is required for its function (Sladek *et al.*, 1990).

20 It has been observed that there is an apparent contradiction between the molecular mass of HNF4 $\alpha$  predicted from the primary sequence (50.6 kDa) (Sladek *et al.*, 1990) and that determined by gel electrophoresis (54 kDa) suggesting that this difference may be due to post-translational modification(s). Of the many types of post-translational modifications that might regulate gene expression, most attention has been focused on phosphorylation, which can influence transcription factor activity in many ways (Hunter and Karin, 1992).

30 Three main levels of regulation have been described: phosphorylation can affect the DNA-binding activity (Boyle *et al.*, 1991; Segil *et al.*, 1991; Shuai *et al.*, 1994), the

transcriptional activation potential (Yamamoto *et al.*, 1988; Trautwein *et al.*, 1993), or the translocation of a transcription factor from the cytoplasm into the nucleus (Metz and Ziff, 1991; Kerr *et al.*, 1991; Schindler *et al.*, 1992; Shuai *et al.*, 1992). These possibilities are by no means mutually exclusive, and in principle phosphorylation can be responsible for 5 simultaneous regulation at several distinct levels. With the exception of certain signal transduction proteins (Darnell *et al.*, 1994), all examples of this type of regulation have involved phosphorylation at serine or threonine residues.

It has been demonstrated that the activity of HNF4 $\alpha$  is post-translationally 10 regulated by tyrosine phosphorylation, providing an example of a non-signal-transduction factor modulated by this modification. The HNF4 $\alpha$  polypeptide (SEQ ID NO:79) contains 12 tyrosine residues scattered throughout the DNA-binding, dimerization, and putative ligand-binding domains (Sladek *et al.*, 1990) which could be potential phosphorylation sites. It seems that the tyrosine phosphorylation of HNF4 $\alpha$  is required 15 for its DNA-binding activity. It has been shown that the transcriptionally active form of HNF4 $\alpha$  is localized in specific subnuclear domains. This intranuclear distribution depends directly or indirectly on tyrosine phosphorylation, suggesting the existence of an additional control mechanism at the level of subnuclear targeting playing a role in transcription regulation.

20 Hepatocyte nuclear factor 4 $\alpha$  (HNF-4 $\alpha$ ) is a positive-acting transcription factor which is expressed very early in embryo development and is essential to liver development and function (reviewed in Sladek, 1993 and Sladek, 1994). Mouse HNF4 $\alpha$  mRNA appears in the primary endoderm of implanting blastocysts at embryonic day 4.5 25 and in the liver and gut primordia at day 8.5 (Duncan *et al.*, 1994), while mice deficient in HNF4 $\alpha$  do not survive past day 9 postcoitus (Chen *et al.*, 1994).

HNF4 $\alpha$  has also been proposed to be responsible for the final commitment for cells to differentiate into hepatocytes (Nagy *et al.*, 1994). In adult rodents, HNF4 $\alpha$  is

located primarily in the liver, kidney, and intestine, and in insects HNF4 $\alpha$  is found in the equivalent tissues (Sladek *et al.*, 1990; Zhong *et al.*, 1993). HNF4 $\alpha$  is known to activate a wide variety of essential genes, including those involved in cholesterol, fatty acid, and glucose metabolism; blood coagulation; detoxification mechanisms; hepatitis B virus infections; and liver differentiation (reviewed in Sladek, 1993 and Sladek, 1994).

HNF4 $\alpha$  is a member of the superfamily of ligand-dependent transcription factors, which includes the steroid hormone receptors, thyroid hormone receptor (TR), vitamin A receptor, and vitamin D receptor (VDR), as well as a large number of receptors for which 10 ligands have not yet been identified, the so-called orphan receptors (reviewed in Landers and Spelsberg, 1992; O'Malley and Conneely, 1992; Parker, 1993; and Tsai and O'Malley, 1994). All receptors are characterized by two conserved domains: the zinc finger region, which mediates DNA binding, and a large hydrophobic domain which mediates protein dimerization, transactivation, and ligand binding.

15

Whether HNF4 $\alpha$  responds to a ligand is not known, but it has been shown to activate transcription in the absence of an exogenously added ligand (Hall *et al.*, 1994; Kuo *et al.*, 1992; Metzger *et al.*, 1993; Mietus *et al.*, 1992; Reijnen *et al.*, 1992; Sladek *et al.*, 1990). HNF4 $\alpha$  is also highly conserved with the *Drosophila* HNF-4, containing 91% 20 amino acid sequence identity to the rat HNF4 $\alpha$  in the DNA binding domain and 68% identity in the large hydrophobic domain (Zhong *et al.*, 1993).

The members of the receptor superfamily have been classified in a variety of ways, one of which is by their ability to dimerize with themselves and with other 25 members of the superfamily. For example, the steroid hormone receptors, glucocorticoid, mineralocorticoid, and progesterone receptors (GR, MR, and PR, respectively), all bind DNA and activate transcription as homodimers. They are present in the cytoplasm complexed with heat shock proteins (HSP) until the presence of the appropriate ligand disrupts the complex, allowing the receptors to translocate to the nucleus (reviewed in Freedman and Luisi, 1993; O'Malley and Tsai, 1993; and Tsai and O'Malley, 1994). On 30

the other hand, the retinoid acid receptor (RAR) and retinoid X receptor (RXR) as well as the VDR, peroxisome proliferator-activated receptor (PPAR), and TR, which do not bind HSP and reside primarily in the nucleus, all bind DNA and activate transcription not only as homodimers but also as heterodimers (reviewed in Giguère, 1994; Parker, 1993; and Stunnenberg, 1993). Several of the nuclear receptors bind DNA very inefficiently, if at all, as homodimers (RXR $\alpha$ , RAR, VDR, TR, and PPAR) but bind DNA well as heterodimers (reviewed in Giguère, 1994 and Stunnenberg, 1993). At least two of the receptors (RAR and TR) form heterodimers in solution with RXR $\alpha$  (Hermann *et al.*, 1992; Kurokawa *et al.*, 1993; Zhang *et al.*, 1992).

10

The most common dimerization partner for all of these receptors is RXR $\alpha$ . The third class of receptors identified to date reside in both the nucleus and the cytoplasm and bind DNA preferentially as monomers (NGFI-B, FTZ-F1, steroidogenic factor 1 [SF-1], and ROR $\alpha$ 1) (Giguère *et al.*, 1995; Kurachi *et al.*, 1994; Ohno *et al.*, 1994).

15

HNF4 $\alpha$  is very similar to the retinoid receptors, in particular to RXR $\alpha$ , in both amino acid sequence and DNA binding specificity. Mouse RXR $\alpha$  is 60% identical to rat HNF4 $\alpha$  in the DNA binding domain and 44% identical in the large hydrophobic domain. In comparison, RAR $\alpha$ , which readily heterodimerizes with RXR $\alpha$ , is 61% identical to RXR $\alpha$  in the DNA binding domain and only 27% identical in the large hydrophobic domain (Mangelsdorf *et al.*, 1992). HNF4 $\alpha$  and RXR $\alpha$  have also been shown to share response elements from at least six different genes as well as a consensus site of a direct repeat of AGGTCA separated by one nucleotide (referred to as DR+1) (Carter *et al.*, 1994; Carter *et al.*, 1993; Garcia *et al.*, 1993; Ge *et al.*, 1994; Hall *et al.*, 1994; Hall *et al.*, 1992; Kekule *et al.*, 1993; Ladias, 1994; Lucas *et al.*, 1991; Nakshatri and Chambon, 1994; Widom *et al.*, 1992). The structural and functional similarities of HNF4 $\alpha$  and RXR $\alpha$  suggest that HNF4 $\alpha$  might heterodimerize with RXR $\alpha$  and/or other receptors.

Electrophoretic mobility shift analyses (EMSA) of HNF4 $\alpha$  and RXR $\alpha$  proteins expressed *in vivo* and *in vitro* showed that HNF4 $\alpha$  in fact does not heterodimerize with RXR $\alpha$  on any one of a number of response elements and that while HNF4 $\alpha$  forms homodimers in solution in the absence the DNA, it does not form heterodimers with 5 RXR $\alpha$ . It has also been shown that HNF4 $\alpha$  does not heterodimerize with a number of other receptors on DNA, suggesting that the lack of heterodimerization is a general property of HNF4 $\alpha$ .

These studies led to the proposal that HNF4 $\alpha$  defines a new subfamily of nuclear 10 receptors which are presently exclusively in the nucleus, exist in solution, bind DNA as homodimers, and do not form heterodimers with RXR $\alpha$  or other receptors.

HNF4 $\alpha$  is a member of the steroid hormone receptor family. The members of this family have been classified according to the amino acid sequence in the knuckle of the 15 first zinc finger (referred to as the P box) a region important for recognizing the sequence of the half site of the palindrome in hormone response elements (Forman and Samuels, 1990). For examples members of the thyroid hormone receptor subfamily contain amino acid sequence EGCKG (SEQ ID NO:83) and bind to the thyroid response element (TRE). Members of the estrogen receptor subfamily contain the amino acids EGCKA (SEQ ID 20 NO:84) and bind to estrogen response elements (ERE). The sequence of HNF4 $\alpha$  is DGCKG (SEQ ID NO:85) and is most similar to that of the thyroid response element. Despite this similarity it appears that HNF4 $\alpha$  does not bind TRE nor does it bind ERE, and the true ligand for HNF4 $\alpha$  is as yet undetermined. The screening methods of the present invention will lead one of ordinary skill in the art to elucidate such a ligand or 25 ligands.

The present invention describes the exon-intron organization and partial sequence of the human HNF4 $\alpha$  gene. In addition, the inventors have screened the exons, flanking introns and minimal promoter region for mutations in a group of 57 unrelated Japanese

subjects with early-onset diabetes/MODY of unknown cause. The results of these screens suggest that mutations in the HNF4 $\alpha$  gene may cause early-onset diabetes/MODY in Japanese but they are less common than mutations in the HNF1 $\alpha$  /MODY3 gene. The information presented herein on the sequence of the HNF4 $\alpha$  gene and its promoter region  
5 will facilitate the search for mutations in other populations and studies of the role of this gene in determining normal pancreatic  $\beta$ -cell function.

Furthermore, current understanding of the MODY1 form of diabetes is based on studies of only a single family, the R-W pedigree. Here the inventors report the  
10 identification of a second family with MODY1 and the first in which there has been a detailed characterization of hepatic function. The present inventors demonstrate that MODY1 is primarily a disorder of  $\beta$ -cell function, however, the inventors have ascertained that mutations in HNF4 $\alpha$  may lead to  $\alpha$ -cell as well as  $\beta$ -cell secretory defects or to a reduction in pancreatic islet mass.  
15

#### *Hepatic Nuclear Factor 1 $\beta$ and DCoH*

Human HNF1 $\beta$  is a homeodomain-containing transcription factor of 557 amino acids (type A) with alternative splicing generating two other forms of 531 (type B) and 399 amino acids (type C) (Mendel *et al.*, 1991a; De Simone *et al.*, 1991; Rey-Campos *et al.*, 1991; Bach and Yaniv, 1993). The nucleic and amino acid sequences for human HNF1 $\beta$  are given in SEQ ID NO:128 and SEQ ID NO:129, respectively. HNF1 $\beta$  is structurally related to HNF1 $\alpha$  and functions as a homodimer or a heterodimer with HNF1 $\alpha$ . These dimers are stabilized by the bifunctional protein, DCoH/PCBD (Mendel *et al.*, 1991b; Citron *et al.*, 1992), which binds to the dimerization domain of HNF1 forming a heterotetrameric complex and enhancing transcriptional activity. As a homotetramer, PCBD is involved in the regeneration of tetrahydrobiopterin, an essential cofactor of phenylalanine hydroxylase and other mono-oxygenases, catalyzing the conversion of 4-hydroxytetrahydrobiopterin to quinonoid-dihydrobiopterin (Citron *et al.*, 1993; Johnen *et al.*, 1995). Loss of function mutations in PCBD are associated with a  
20  
25

rare autosomal recessive form of mild hyperphenylalaninemia. HNF1 $\beta$  and DCoH mRNA are expressed in mouse pancreatic islets implying that they may function together with HNF-1 $\alpha$  to regulate gene expression in this tissue. Human DCoH is a protein of 104 amino acids (including the initiating methionine) (Thöny *et al.*, 1995) and functions as  
5 described herein below.

***MODY-type Diabetes is a Manifestation of Defects in Hepatocyte Nuclear Factors***

It is established that all forms of Type 2 diabetes are associated with profound  
10 insulin secretory defects which include loss of the first phase response to intravenous glucose, delayed and blunted responses to ingestion of a mixed meal, loss of the normal oscillatory patterns of insulin secretion, and increased secretion of proinsulin and proinsulin-like products. The molecular basis of these secretory defects in humans is unknown, although in rats it has been shown that there are global changes in gene  
15 expression in the islets of diabetic and prediabetic animals. One such global alteration is the reduction in the levels of mRNAs encoding many pancreatic islet specific proteins. This defect in gene expression would be compatible with decreased levels of a master transcription factor whose levels affect the expression of a whole array of downstream genes.  
20

The present invention predicts that the  $\beta$ -cell dysfunction and insulin secretory defects associated with MODY3 are as a result of mutations in HNF1 $\alpha$ , furthermore it demonstrates that  $\beta$ -cell dysfunction associated with MODY1 are a result of mutations in HNF4 $\alpha$ .  
25

The features of MODY-type diabetes are very similar to those of late onset Type 2 diabetes. Hence, acquired defects in the expression of HNF1 $\alpha$ , HNF4 $\alpha$ , and HNF1 $\beta$ , respectively, may well occur in late onset diabetes and lead to  $\beta$ -cell dysfunction and insulin secretory defects in this form of diabetes. The identification of agents that

activate transcription of HNF1 $\alpha$ , HNF1 $\beta$  and HNF4 $\alpha$  will be therapeutic for the treatment of MODY, as well as late onset Type 2 diabetes. The present invention details methods for the identification of such agents which will then be used to increase the expression of HNF1 $\alpha$ , HNF1 $\beta$  and HNF4 $\alpha$  which in turn will lead to the increased  
5 transcription/expression or activation of  $\beta$ -cell genes such as insulin.

It is clear from the present invention that hepatocyte nuclear factors, their expression, regulation and modification have far reaching implications in diabetes. To date three of the four types of MODY diabetes identified, are predicted to affect gene  
10 expression. Other forms of MODY can not be ruled out, for example genetic linkage studies predict the presence of additional MODY genes, the chromosomal localization of which are presently unknown.

The absolute HNF4 $\alpha$  dependence of the HNF1 $\alpha$  promoter coupled with evidence  
15 of the ability of HNF4 $\alpha$  to rescue endogenous HNF1 $\alpha$  expression is indicative of HNF4 $\alpha$  being an essential regulator of HNF1 $\alpha$  (FIG. 6). Thus activation or repression of HNF4 $\alpha$  will result in an indirect activation or repression of HNF1 $\alpha$ . The present invention elucidates methods for identifying factors responsible for modulating HNF4 $\alpha$  expression and/or activity.  
20

HNF1 $\beta$ , also known as vHNF1, is closely related to HNF1 $\alpha$  and is able to form heterodimers with HNF1 $\alpha$ . Dimerization between members of classes of transcription factors appears to solve the problem of controlling expression of a very large number genes. An obvious advantage of the dimerization ability of a transcription factor is that it  
25 provides an opportunity to diversify the number of regulatory mechanisms that can be associated with a single regulatory DNA binding site. Another advantage lies in the possibility of translating subtle alterations in the relative levels of expression of members of a dimerization pair into a substantial quantitative effect on transcription.

FIG. 6 summarizes the different factors involved in the regulation of expression and activity of the HNF transcription factors described above. From the inventors investigations it is conceivable that aberrations at any points along this pathway or any factors affecting this pathway directly or indirectly will result in β-cell dysfunction and diabetes mellitus, either as MODY or late-onset diabetes.

The present invention has shown that mutations in HNF1α are clearly responsible for MODY3 type diabetes. As discussed earlier HNF1α binds to DNA as a dimer. this can either be a homodimer or a heterodimer with HNF1β (SEQ ID NO: 80). The two forms of HNF1 are expressed in comparable amounts in the liver but there is a three-fold higher expression of HNF1β in the kidney as compared to HNF1α.

HNF1β lacks the transcriptional activity attributable to HNF1α. One potential consequence of this observation in combination with its ability to dimerize with HNF1α is that HNF1β is likely to be a negative regulator of HNF1α transcriptional activity. This observation is suggested by the presence of vHNF1 in systems that do not express the majority of hepatocyte-specific gene products (Baumhueter *et al.*, 1988). However, studies by Mendel *et al.*, (1991) were unable to confirm this observation.

Studies by Mendel *et al.*, (1991) indicated that a dimerization cofactor of HNF1 (DCoH) may increase the stability of HNF1α dimers. Thus, it is suggested that DCoH has the potential to restrict the activity of HNF1α and/or HNF1β. There are a number of hypothesis as to how DCoH affects HNF1 activation of transcription. HNF1α is a monomer in solution and can only bind DNA as a dimer, the presence of DCoH favors the formation of the dimeric HNF1α. Alternatively it is plausible that DCoH induces a conformational change in HNF1α to create a more potent transcriptional activator either directly or by allowing interaction with other proteins, for example HNF1β. Yet another alternative is that DCoH decreases the rate of HNF1α degradation thereby stabilizing HNF1α and potentiating the effects of HNF1α.

The present invention demonstrates that MODY4, which was previously uncharacterized, is a manifestation of defects in HNF1 $\beta$ . The present invention describes specific mutations in HNF1 $\beta$  that have led to MODY4 in certain individuals. In light of 5 these observations, there are described herein methods for the identification and isolation of factors involved in the activity of HNF1 $\beta$  and DCoH with a view to obtaining insights into therapeutic intervention in diabetes.

### C. *In vitro* Screening Assays for Candidate Substances

10 Certain aspects of this invention concern methods for conveniently evaluating candidate substances to identify compounds capable of stimulating HNF1 $\alpha$ -, HNF1 $\beta$ - or HNF4 $\alpha$ -mediated transcription. Such compounds will be capable of promoting gene expression, and thus can be said to have up-regulating activity. In as much as increased gene expression of, for example, the insulin gene in the body functions to alleviate the 15 symptoms of diabetes, any positive substances identified by the assays of the present invention will be anti-diabetic drugs. Before human administration, such compounds would be rigorously tested using conventional animal models known to those of skill in the art.

20 Successful candidate substances may function in the absence of mutations in HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  in which case the candidate compound may be termed a "positive stimulator" of HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$ , respectively. Alternatively, such compounds may stimulate transcription in the presence of mutated HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  overcoming the effects of the mutations, i.e., function to oppose HNF1 $\alpha$ -mutant, 25 and/or HNF1 $\beta$ , and/or HNF4 $\alpha$ -mediated diabetes, and thus may be termed "an HNF1 $\alpha$  mutant agonist" "HNF1 $\beta$  mutant agonist" or "HNF4 $\alpha$  mutant agonist" respectively. Compounds may even be discovered which combine all three of these actions. Although the agonist class of compounds may ultimately seem to be the most desirable, compounds of either class will likely be useful therapeutic agents for use in stimulating gene

expression and combating MODY1, MODY3, MODY4, and late-onset Type 2 diabetes in human subjects.

*Candidates for HNF1 $\alpha$*

As HNF1 $\alpha$  is herein shown to be linked to MODY3 type, one method by which to identify a candidate substance capable of stimulating *HNF1 $\alpha$* -mediated transcription in diabetes is based upon specific protein:DNA binding. Accordingly, to conduct such an assay, one may prepare an *HNF1 $\alpha$*  binding protein composition, such as recombinant *HNF1 $\alpha$* , and determine the ability of a candidate substance to increase *HNF1 $\alpha$*  protein binding to a DNA segment including a complementary *HNF1 $\alpha$*  binding sequence, i.e., to increase the amount or the binding affinity of a protein:DNA complex.

This generally would be achieved using two parallel assays, one of which contains HNF1 $\alpha$  and the specific DNA alone and one of which contains HNF1 $\alpha$ , DNA and the candidate substance composition. One would perform each assay under conditions, and for a period of time, effective to allow the formation of protein:DNA complexes, and one would then separate the bound protein:DNA complexes from any unbound protein or DNA and measure the amount of the protein:DNA complexes. An increase in the amount of the bound protein:DNA complex formed in the presence of the candidate substance would be indicative of a candidate substance capable of promoting HNF1 $\alpha$  binding, and thus, capable of stimulating HNF1 $\alpha$ -mediated transcription.

In such binding assays, the amount of the protein:DNA complex may be measured, after the removal of unbound species, by detecting a label, such as a radioactive or enzymatic label, which has been incorporated into the original HNF1 $\alpha$  protein composition or recombinant protein or HNF1 $\alpha$ -containing DNA segment. Alternatively, one could detect the protein portion of the complex by means of an antibody directed against the protein, such as those disclosed herein.

Preferred binding assays are those in which either the HNF1 $\alpha$  protein, recombinant protein or purified composition or the HNF1 $\alpha$ -containing DNA segment is bound to a solid support and contacted with the other component to allow complex formation. Unbound protein or DNA components are then separated from the 5 protein:DNA complexes by washing and the amount of the remaining bound complex quantitated by detecting the label or with antibodies. Such DNA binding assays form the basis of filter-binding and microtiter plate-type assays and can be performed in a semi- automated manner to enable analysis of a large number of candidate substances in a short period of time. Electrophoretic methods, such as the gel-shift assay disclosed herein, 10 could also be employed to separate unbound protein or DNA from bound protein:DNA complexes, but such labor-intensive methods are not preferred.

Assays such as those described above are initially directed to identifying positive stimulator candidate substances and do not, by themselves, address the activity of the 15 substance in the presence of HNF1 $\alpha$  mutants. However, such positive regulators may also prove to act as HNF1 $\alpha$  mutant agonists, and in any event, would likely have utility in transcriptional promotion, either *in vitro* or *in vivo*. Positive regulators would likely be further evaluated to assess the effects of HNF1 $\alpha$  mutants on their action, for example, by employing a cellular reporter gene assay such as those described herein below.

20 Virtually any candidate substance may be analyzed by these methods, including compounds which may interact with HNF1 $\alpha$  binding protein(s), HNF1 $\alpha$  or protein:DNA complexes, and also substances such as enzymes which may act by physically altering one of the structures present. Of course, any compound isolated from natural sources 25 such as plants, animals or even marine, forest or soil samples, may be assayed, as may any synthetic chemical or recombinant protein.

Another potential method for stimulating HNF1 $\alpha$ -mediated transcription is to prepare a HNF1 $\alpha$  protein composition and to modify the protein composition in a manner

effective to increase HNF1 $\alpha$  protein binding to a DNA segment including the HNF1 $\alpha$  protein binding sequence. The binding assays would be performed in parallel, similar to those described above, allowing the native and modified HNF1 $\alpha$  binding protein to be compared. In addition to phosphatases and kinases, other agents, including proteases and  
5 chemical agents, could be employed to modify HNF1 $\alpha$  binding protein. The present invention, with the cloning of mutant HNF1 $\alpha$  cDNA, also opens the way for genetically engineering HNF1 $\alpha$  protein to promote gene transcription in diabetes. In this regard, the mutation of potential phosphorylation sites and/or the modification or deletion of other domains is contemplated.

10

*Candidates for HNF4 $\alpha$  binding*

The criteria shown above for screening of modulators of HNF1 $\alpha$  are also true of HNF4 $\alpha$ . HNF4 $\alpha$  is a member of the steroid hormone receptor superfamily however, the ligand for HNF4 $\alpha$  is unknown. The identification of the endogenous ligand for HNF4 $\alpha$   
15 binding would be an important step towards elucidating the mechanisms of eukaryotic gene control, and would also provide biomedical science with a powerful tool by which to regulate specific gene expression. Such a development would lead to numerous useful applications in the pharmaceutical and biotechnological industries. Although many applications are envisioned, one particularly useful application would be as the central  
20 component in screening assays to identify new classes of pharmacologically active substances which may be employed to manipulate, and particularly, to promote, the transcription of genes whose expression is altered in diabetes.

Hence HNF4 $\alpha$  would be of great use in identifying agents to combat MODY and  
25 Type 2 diabetes. An anti-diabetic agent isolated by the screening methods of the present invention would act to promote the cellular transcription or function of HNF4 $\alpha$ , which would in turn serve to increase transcription of genes whose activity is regulated by HNF4 $\alpha$  (for example HNF1 $\alpha$ ) thereby increasing the transcription of genes involved in diabetes and alleviating the symptoms of diabetes.

### *Candidates for HNF1 $\beta$ binding*

The criteria shown above for screening of modulators of HNF1 $\alpha$  and HNF4 $\alpha$  are also true of HNF1 $\beta$ . HNF1 $\beta$  is a 557 amino acid that is structurally related to HNF1 $\alpha$  and functions as a homodimer and heterodimer with HNF1 $\alpha$ . These dimers are stabilized by DCoH. The identification of factors that affect this dimerization, or any of the factors involved in the heterotetrameric complex, will provide useful compounds for the modulation of transcriptional activity. Such a development would lead to numerous useful applications in the pharmaceutical and biotechnological industries. Although many applications are envisioned, one particularly useful application would be as the central component in screening assays to identify new classes of pharmacologically active substances which may be employed to manipulate, and particularly, to promote, the transcription of genes whose expression is altered in diabetes.

Hence HNF1 $\beta$  would be of great use in identifying agents to combat MODY and Type 2 diabetes. An anti-diabetic agent isolated by the screening methods of the present invention would act to promote the cellular transcription or function of HNF1 $\beta$ , which would in turn serve to increase transcription of genes whose activity is regulated by HNF1 $\beta$  (for example HNF1 $\alpha$ ) thereby increasing the transcription of genes involved in diabetes and alleviating the symptoms of diabetes.

### **D. Reporter Genes and Cell-Based Screening Assays**

Cellular assays also are available for screening candidate substances to identify those capable of stimulating HNF1 $\alpha$ - HNF1 $\beta$ - and HNF4 $\alpha$ -mediated transcription and gene expression. In these assays, the increased expression of any natural or heterologous gene under the control of a functional HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  protein may be employed as a measure of stimulatory activity, although the use of reporter genes is preferred. A reporter gene is a gene that confers on its recombinant host cell a readily detectable phenotype that emerges only under specific conditions. In the present case, the

reporter gene, being under the control of a functional HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  protein, will generally be repressed under conditions of MODY3, MODY4 or MODY1 diabetes respectively and will generally be expressed in the MODY3, MODY4 or MODY1 non diabetic conditions respectively.

5

Reporter genes are genes which encode a polypeptide not otherwise produced by the host cell which is detectable by analysis of the cell culture, e.g., by fluorometric, radioisotopic or spectrophotometric analysis of the cell culture. Exemplary enzymes include luciferases, transferases, esterases, phosphatases, proteases (tissue plasminogen activator or urokinase), and other enzymes capable of being detected by their physical presence or functional activity. A reporter gene often used is chloramphenicol acetyltransferase (CAT) which may be employed with a radiolabeled substrate, or luciferase, which is measured fluorometrically.

15

Another class of reporter genes which confer detectable characteristics on a host cell are those which encode polypeptides, generally enzymes, which render their transformants resistant against toxins, e.g., the *neo* gene which protects host cells against toxic levels of the antibiotic G418, and genes encoding dihydrofolate reductase, which confers resistance to methotrexate. Genes of this class are not generally preferred since the phenotype (resistance) does not provide a convenient or rapid quantitative output. Resistance to antibiotic or toxin requires days of culture to confirm, or complex assay procedures if other than a biological determination is to be made.

25

Other genes of potential for use in screening assays are those capable of transforming hosts to express unique cell surface antigens, e.g., viral *env* proteins such as HIV gp120 or herpes gD, which are readily detectable by immunoassays. However, antigenic reporters are not preferred because, unlike enzymes, they are not catalytic and thus do not amplify their signals.

The polypeptide products of the reporter gene are secreted, intracellular or, as noted above, membrane bound polypeptides. If the polypeptide is not ordinarily secreted it is fused to a heterologous signal sequence for processing and secretion. In other circumstances the signal is modified in order to remove sequences that interdict secretion.

5 For example, the herpes gD coat protein has been modified by site directed deletion of its transmembrane binding domain, thereby facilitating its secretion (EP 139,417A). This truncated form of the herpes gD protein is detectable in the culture medium by conventional immunoassays. Preferably, however, the products of the reporter gene are lodged in the intracellular or membrane compartments. Then they can be fixed to the  
10 culture container, *e.g.*, microtiter wells, in which they are grown, followed by addition of a detectable signal generating substance such as a chromogenic substrate for reporter enzymes.

15 The transcriptional promotion process which, in its entirety, leads to enhanced transcription is termed "activation." The mechanism by which a successful candidate substance acts is not material since the objective is to promote HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  mediated gene expression, or even, to promote gene expression in the presence of mutant HNF1 $\alpha$ , HNF1 $\beta$ , or HNF4 $\alpha$  gene products, by whatever means.

20 To create an appropriate vector or plasmid for use in such assays one would ligate the HNF1 $\alpha$ -containing promoter, whether a hybrid or the native HNF1 $\alpha$  promoter, to a DNA segment encoding the reporter gene by conventional methods. Similar assays are also contemplated using HNF1 $\beta$  and HNF4 $\alpha$  promoters. The HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  promoter sequences may be obtained by *in vitro* synthesis or recovered from genomic  
25 DNA and should be ligated upstream of the start codon of the reporter gene. The present invention provides the promoter region for human HNF1 $\alpha$ , a comparison of the sequence of the promoter region of the human, rat, mouse, chicken and frog HNF1 $\alpha$  genes is given in FIG. 22. There is also provided herein a comparison of the sequences of the promoter regions of the human and mouse HNF4 $\alpha$  genes (FIG. 13). The partial sequence of the

human HNF1 $\beta$  gene including promoter has also been identified by the present inventors and deposited in the GenBank database under accession numbers U90279-90287 and U96079. Any of these promoters may be particularly preferred in the present invention. An AT-rich TATA box region should also be employed and should be located between  
5 the HNF sequence and the reporter gene start codon. The region 3' to the coding sequence for the reporter gene will ideally contain a transcription termination and polyadenylation site. The promoter and reporter gene may be inserted into a replicable vector and transfected into a cloning host such as *E. coli*, the host cultured and the replicated vector recovered in order to prepare sufficient quantities of the construction for  
10 later transfection into a suitable eukaryotic host.

Host cells for use in the screening assays of the present invention will generally be mammalian cells, and are preferably cell lines which may be used in connection with transient transfection studies. Cell lines should be relatively easy to grow in large scale  
15 culture. Also, they should contain as little native background as possible considering the nature of the reporter polypeptide. Examples include the Hep G2, VERO, HeLa, human embryonic kidney (HEK)- 293, CHO, WI38, BHK, COS-7, and MDCK cell lines, with monkey CV-1 cells being particularly preferred.

The screening assay typically is conducted by growing recombinant host cells in  
20 the presence and absence of candidate substances and determining the amount or the activity of the reporter gene. To assay for candidate substances capable of exerting their effects in the presence of mutated HNF1 $\alpha$ , HNF1 $\beta$  and/or HNF4 $\alpha$  gene products, one would make serial molar proportions of such gene products that alter HNF1 $\alpha$ -, HNF1 $\beta$ -  
25 and HNF4 $\alpha$ -mediated expression. One would ideally measure the reporter signal level after an incubation period that is sufficient to demonstrate mutant-mediated repression of signal expression in controls incubated solely with mutants. Cells containing varying proportions of candidate substances would then be evaluated for signal activation in comparison to the suppressed levels.

Candidates that demonstrate dose related enhancement of reporter gene transcription or expression are then selected for further evaluation as clinical therapeutic agents. The stimulation of transcription may be observed in the absence of mutant HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$ , in which case the candidate compound might be a positive  
5 stimulator of HNF1 $\alpha$  HNF1 $\beta$  or HNF4 $\alpha$  transcription, respectively. Alternatively, the candidate compound might only give a stimulation in the presence mutated HNF1 $\alpha$ , mutated HNF1 $\beta$  or mutated HNF4 $\alpha$  protein, which would indicate that it functions to oppose the mutation-mediated suppression of the gene expression. Candidate compounds of either class might be useful therapeutic agents that would stimulate gene expression  
10 and thereby combating MODY and Type 2 diabetes.

#### E. Nucleic Acids

As described the Examples, the present invention discloses the gene at the MODY3 locus of chromosome 12, MODY4 locus as being associated with HNF1 $\beta$  and the gene at  
15 the MODY1 locus of chromosome 20. Mutations in these genes are responsible for diabetes. The present invention discloses mutations in the HNF1 $\alpha$ , HNF1 $\beta$ , and HNF4 $\alpha$  genes identified by PCR techniques. The gene for the MODY3 locus has for the first time been identified as hepatocyte nuclear factor 1 $\alpha$ , herein referred to as HNF1 $\alpha$ . The gene for the MODY1 locus has been identified as hepatocyte nuclear factor 4  $\alpha$  (HNF4 $\alpha$ ). The gene  
20 for the MODY4 locus has been identified as hepatocyte nuclear factor 1 $\beta$  (HNF1 $\beta$ ).

In one embodiment of the present invention, the nucleic acid sequences disclosed herein find utility as hybridization probes or amplification primers. In certain embodiments, these probes and primers consist of oligonucleotide fragments. Such fragments should be  
25 of sufficient length to provide specific hybridization to an RNA or DNA sample extracted from tissue. The sequences typically will be 10-20 nucleotides, but may be longer. Longer sequences, *e.g.*, 40, 50, 100, 500 and even up to full length, are preferred for certain embodiments.

Nucleic acid molecules having contiguous stretches of about 10, 15, 17, 20, 30, 40, 50, 60, 75 or 100 or 500 nucleotides from a sequence selected from the group comprising SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, HNF1 $\alpha$  and its mutants are contemplated. In other embodiments nucleotides from a sequence selected from the group comprising SEQ ID NO:78, SEQ ID NO:34, SEQ ID NO:36, SEQ ID NO:38, SEQ ID NO:40, SEQ ID NO:42, SEQ ID NO:44, SEQ ID NO:46, SEQ ID NO:48, SEQ ID NO:50, SEQ ID NO:52, SEQ ID NO:54, HNF4 $\alpha$  and its mutants are contemplated. In still other embodiments nucleotides from a sequence selected from the group comprising SEQ ID NO:---, HNF1 $\beta$  and its mutants are contemplated. Molecules that are complementary to the above mentioned sequences and that bind to these sequences under high stringency conditions also are contemplated. These probes will be useful in a variety of hybridization embodiments, such as Southern and northern blotting. In some cases, it is contemplated that probes may be used that hybridize to multiple target sequences without compromising their ability to effectively diagnose diabetes (MODY1, MODY3, and MODY4). In certain embodiments, it is contemplated that multiple probes may be used for hybridization to a single sample.

Various probes and primers can be designed around the disclosed nucleotide sequences. Primers may be of any length but, typically, are 10-20 bases in length. By assigning numeric values to a sequence, for example, the first residue is 1, the second residue is 2, etc., an algorithm defining all primers can be proposed:

25 n to n + y

where n is an integer from 1 to the last number of the sequence and y is the length of the primer minus one, where n + y does not exceed the last number of the sequence. Thus, for a 10-mer, the probes correspond to bases 1 to 10, 2 to 11, 3 to 12 ... and so on. For a 15-

mer, the probes correspond to bases 1 to 15, 2 to 16, 3 to 17 ... and so on. For a 20-mer, the probes correspond to bases 1 to 20, 2 to 21, 3 to 22 ... and so on.

The values of n in the algorithm above for the nucleic acid sequences is: SEQ ID  
5 NO:1, n=3238 for HNF1 $\alpha$ , SEQ ID NO:78 n=1441 for HNF4 $\alpha$ , SEQ ID NO:128.

The use of a hybridization probe of between 17 and 100 nucleotides in length allows  
the formation of a duplex molecule that is both stable and selective. Molecules having  
complementary sequences over stretches greater than 20 bases in length are generally  
10 preferred, in order to increase stability and selectivity of the hybrid, and thereby improve the  
quality and degree of particular hybrid molecules obtained. One will generally prefer to  
design nucleic acid molecules having stretches of 20 to 30 nucleotides, or even longer  
where desired. Such fragments may be readily prepared by, for example, directly  
synthesizing the fragment by chemical means or by introducing selected sequences into  
15 recombinant vectors for recombinant production.

Accordingly, the nucleotide sequences of the invention may be used for their ability  
to selectively form duplex molecules with complementary stretches of genes or RNAs or to  
provide primers for amplification of DNA or RNA from tissues. Depending on the  
20 application envisioned, one will desire to employ varying conditions of hybridization to  
achieve varying degrees of selectivity of probe towards target sequence.

For applications requiring high selectivity, one will typically desire to employ  
relatively stringent conditions to form the hybrids, *e.g.*, one will select relatively low salt  
25 and/or high temperature conditions, such as provided by about 0.02 M to about 0.10 M  
NaCl at temperatures of about 50°C to about 70°C. Such high stringency conditions tolerate  
little, if any, mismatch between the probe and the template or target strand, and would be  
particularly suitable for isolating specific genes or detecting specific mRNA transcripts. It  
is generally appreciated that conditions can be rendered more stringent by the addition of  
30 increasing amounts of formamide.

For certain applications, for example, substitution of nucleotides by site-directed mutagenesis, it is appreciated that lower stringency conditions are required. Under these conditions, hybridization may occur even though the sequences of probe and target strand  
5 are not perfectly complementary, but are mismatched at one or more positions. Conditions may be rendered less stringent by increasing salt concentration and decreasing temperature. For example, a medium stringency condition could be provided by about 0.1 to 0.25 M NaCl at temperatures of about 37°C to about 55°C, while a low stringency condition could be provided by about 0.15 M to about 0.9 M salt, at temperatures ranging from about 20°C  
10 to about 55°C. Thus, hybridization conditions can be readily manipulated depending on the desired results.

In other embodiments, hybridization may be achieved under conditions of, for example, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl<sub>2</sub>, 1.0 mM dithiothreitol, at  
15 temperatures between approximately 20°C to about 37°C. Other hybridization conditions utilized could include approximately 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, at temperatures ranging from approximately 40°C to about 72°C.

In certain embodiments, it will be advantageous to employ nucleic acid sequences of  
20 the present invention in combination with an appropriate means, such as a label, for determining hybridization. A wide variety of appropriate indicator means are known in the art, including fluorescent, radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of being detected. In preferred embodiments, one may desire to employ a fluorescent label or an enzyme tag such as urease, alkaline phosphatase or peroxidase,  
25 instead of radioactive or other environmentally undesirable reagents. In the case of enzyme tags, colorimetric indicator substrates are known that can be employed to provide a detection means visible to the human eye or spectrophotometrically, to identify specific hybridization with complementary nucleic acid-containing samples.

In general, it is envisioned that the hybridization probes described herein will be useful both as reagents in solution hybridization, as in PCR, for detection of expression of corresponding genes, as well as in embodiments employing a solid phase. In embodiments involving a solid phase, the test DNA (or RNA) is adsorbed or otherwise affixed to a selected matrix or surface. This fixed, single-stranded nucleic acid is then subjected to hybridization with selected probes under desired conditions. The selected conditions will depend on the particular circumstances based on the particular criteria required (depending, for example, on the G+C content, type of target nucleic acid, source of nucleic acid, size of hybridization probe, *etc.*). Following washing of the hybridized surface to remove non-specifically bound probe molecules, hybridization is detected, or even quantified, by means of the label.

It will be understood that this invention is not limited to the particular probes disclosed herein and particularly is intended to encompass at least nucleic acid sequences that are hybridizable to the disclosed sequences or are functional analogs of these sequences.

For applications in which the nucleic acid segments of the present invention are incorporated into vectors, such as plasmids, cosmids or viruses, these segments may be combined with other DNA sequences, such as promoters, polyadenylation signals, restriction enzyme sites, multiple cloning sites, other coding segments, and the like, such that their overall length may vary considerably. It is contemplated that a nucleic acid fragment of almost any length may be employed, with the total length preferably being limited by the ease of preparation and use in the intended recombinant DNA protocol.

25

DNA segments encoding a specific gene may be introduced into recombinant host cells and employed for expressing a specific structural or regulatory protein. Alternatively, through the application of genetic engineering techniques, subportions or derivatives of selected genes may be employed. Upstream regions containing regulatory regions such as

promoter regions may be isolated and subsequently employed for expression of the selected gene.

In an alternative embodiment, the HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  nucleic acids  
5 employed may actually encode antisense constructs that hybridize, under intracellular conditions, to an HNF1 $\alpha$  or HNF $\alpha$  nucleic acid, respectively. The term "antisense construct" is intended to refer to nucleic acids, preferably oligonucleotides, that are complementary to the base sequences of a target DNA or RNA. Antisense oligonucleotides, when introduced into a target cell, specifically bind to their target  
10 nucleic acid and interfere with transcription, RNA processing, transport, translation and/or stability.

Antisense constructs may be designed to bind to the promoter and other control regions, exons, introns or even exon-intron boundaries of a gene. Antisense RNA  
15 constructs, or DNA encoding such antisense RNA's, may be employed to inhibit gene transcription or translation or both within a host cell, either *in vitro* or *in vivo*, such as within a host animal, including a human subject. Nucleic acid sequences which comprise "complementary nucleotides" are those which are capable of base-pairing according to the standard Watson-Crick complementarity rules. That is, the larger purines will base pair  
20 with the smaller pyrimidines to form combinations of guanine paired with cytosine (G:C) and adenine paired with either thymine (A:T), in the case of DNA, or adenine paired with uracil (A:U) in the case of RNA. Inclusion of less common bases such as inosine, 5-methylcytosine, 6-methyladenine, hypoxanthine and others in hybridizing sequences does not interfere with pairing.  
25

As used herein, the terms "complementary" means nucleic acid sequences that are substantially complementary over their entire length and have very few base mismatches. For example, nucleic acid sequences of fifteen bases in length may be termed complementary when they have a complementary nucleotide at thirteen or fourteen  
30 positions with only a single mismatch. Naturally, nucleic acid sequences which are

"completely complementary" will be nucleic acid sequences which are entirely complementary throughout their entire length and have no base mismatches.

Other sequences with lower degrees of homology also are contemplated. For  
5 example, an antisense construct which has limited regions of high homology, but also contains a non-homologous region (*e.g.*, a ribozyme) could be designed. These molecules, though having less than 50% homology, would bind to target sequences under appropriate conditions.

10 While all or part of the HNF1 $\alpha$ , HNF1 $\beta$ , HNF4 $\alpha$  gene sequence may be employed in the context of antisense construction, short oligonucleotides are easier to make and increase *in vivo* accessibility. However, both binding affinity and sequence specificity of an antisense oligonucleotide to its complementary target increases with increasing length. It is contemplated that antisense oligonucleotides of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17,  
15 18, 19, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100 or more base pairs will be used. One can readily determine whether a given antisense nucleic acid is effective at targeting of the corresponding host cell gene simply by testing the constructs *in vitro* to determine whether the endogenous gene's function is affected or whether the expression of related genes having complementary sequences is affected.

20 In certain embodiments, one may wish to employ antisense constructs which include other elements, for example, those which include C-5 propyne pyrimidines. Oligonucleotides which contain C-5 propyne analogues of uridine and cytidine have been shown to bind RNA with high affinity and to be potent antisense inhibitors of gene  
25 expression (Wagner *et al.*, 1993).

Throughout this application, the term "expression construct" is meant to include any type of genetic construct containing a nucleic acid coding for a gene product in which part or all of the nucleic acid encoding sequence is capable of being transcribed. The  
30 transcript may be translated into a protein, but it need not be. Thus, in certain

embodiments, expression includes both transcription of a gene and translation of a RNA into a gene product. In other embodiments, expression only includes transcription of the nucleic acid, for example, to generate antisense constructs.

5       In preferred embodiments, the nucleic acid is under transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrase "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control  
10      RNA polymerase initiation and expression of the gene.

The term promoter will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. Much of the thinking about how promoters are organized derives from analyses of several viral  
15      promoters, including those for the HSV thymidine kinase (tk) and SV40 early transcription units. These studies, augmented by more recent work, have shown that promoters are composed of discrete functional modules, each consisting of approximately 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator or repressor proteins.

20       At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying  
25      the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements  
30      downstream of the start site as well. The spacing between promoter elements frequently

is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to  
5 activate transcription.

The particular promoter that is employed to control the expression of a nucleic acid is not believed to be critical, so long as it is capable of expressing the nucleic acid in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the  
10 nucleic acid coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human or viral promoter. Preferred promoters include those derived from HSV, and HNF1 $\alpha$  (see for example, FIG. 22), HNF1 $\beta$  or HNF4 $\alpha$  promoter (see for example,  
15 FIG. 13). The partial sequence of the human HNF1 $\beta$  gene including promoter has also been identified by the present inventors and deposited in the GenBank database under accession numbers U90279-90287 and U96079 (SEQ ID NO:128). Another preferred embodiment is the tetracycline controlled promoter.

In various other embodiments, the human cytomegalovirus (CMV) immediate  
20 early gene promoter, the SV40 early promoter and the Rous sarcoma virus long terminal repeat can be used to obtain high-level expression of transgenes. The use of other viral or mammalian cellular or bacterial phage promoters which are well-known in the art to achieve expression of a transgene is contemplated as well, provided that the levels of expression are sufficient for a given purpose. Tables 1 and 2 list several  
25 elements/promoters which may be employed, in the context of the present invention, to regulate the expression of a transgene. This list is not intended to be exhaustive of all the possible elements involved in the promotion of transgene expression but, merely, to be exemplary thereof.

Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation. Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization.

15

Additionally any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression of a transgene. Use of a T3, T7 or SP6 cytoplasmic expression system is another possible embodiment. Eukaryotic cells can support cytoplasmic transcription from certain bacterial promoters if the appropriate bacterial polymerase is provided, either as part of the delivery complex or as an additional genetic expression construct.

**TABLE 1**

PROMOTER
Immunoglobulin Heavy Chain
Immunoglobulin Light Chain
T-Cell Receptor
HLA DQ $\alpha$ and DQ $\beta$
$\beta$ -Interferon
Interleukin-2
Interleukin-2 Receptor
MHC Class II 5
MHC Class II HLA-DR $\alpha$
$\beta$ -Actin
Muscle Creatine Kinase
Prealbumin (Transthyretin)
Elastase I
Metallothionein
Collagenase
Albumin Gene
$\alpha$ -Fetoprotein
$\alpha$ -Globin
$\beta$ -Globin
c-fos
c-HA-ras
Insulin
Neural Cell Adhesion Molecule (NCAM)
$\alpha_1$ -Anti-trypsin
H2B (TH2B) Histone
Mouse or Type I Collagen

PROMOTER
Glucose-Regulated Proteins (GRP94 and GRP78)
Rat Growth Hormone
Human Serum Amyloid A (SAA)
Troponin I (TN I)
Platelet-Derived Growth Factor
Duchenne Muscular Dystrophy
SV40
Polyoma
Retroviruses
Papilloma Virus
Hepatitis B Virus
Human Immunodeficiency Virus
Cytomegalovirus
Gibbon Ape Leukemia Virus

**TABLE 2**

Element	Inducer
MT II	Phorbol Ester (TPA) Heavy metals
MMTV (mouse mammary tumor virus)	Glucocorticoids
$\beta$ -Interferon	poly(rI)X poly(rc).
Adenovirus 5 E2	Ela
c-jun	Phorbol Ester (TPA), H <sub>2</sub> O <sub>2</sub>
Collagenase	Phorbol Ester (TPA)
Stromelysin	Phorbol Ester (TPA), IL-1
SV40	Phorbol Ester (TPA)
Murine MX Gene	Interferon, Newcastle Disease Virus
GRP78 Gene	A23187
$\alpha$ -2-Macroglobulin	IL-6
Vimentin	Serum
MHC Class I Gene H-2kB	Interferon
HSP70	Ela, SV40 Large T Antigen
Proliferin	Phorbol Ester-TPA
Tumor Necrosis Factor	FMA
Thyroid Stimulating Hormone $\alpha$ Gene	Thyroid Hormone

5        Use of the baculovirus system will involve high level expression from the powerful polyhedron promoter.

One will typically include a polyadenylation signal to effect proper polyadenylation of the transcript. The nature of the polyadenylation signal is not believed  
10      to be crucial to the successful practice of the invention, and any such sequence may be

employed. Preferred embodiments include the SV40 polyadenylation signal and the bovine growth hormone polyadenylation signal, convenient and known to function well in various target cells. Also contemplated as an element of the expression cassette is a terminator. These elements can serve to enhance message levels and to minimize read  
5 through from the cassette into other sequences.

A specific initiation signal also may be required for efficient translation of coding sequences. These signals include the ATG initiation codon and adjacent sequences. Exogenous translational control signals, including the ATG initiation codon, may need to  
10 be provided. One of ordinary skill in the art would readily be capable of determining this and providing the necessary signals. It is well known that the initiation codon must be "in-frame" with the reading frame of the desired coding sequence to ensure translation of the entire insert. The exogenous translational control signals and initiation codons can be either natural or synthetic. The efficiency of expression may be enhanced by the  
15 inclusion of appropriate transcription enhancer elements (Bittner *et al.*, 1987).

In various embodiments of the invention, the expression construct may comprise a virus or engineered construct derived from a viral genome. The ability of certain viruses to enter cells via receptor-mediated endocytosis and to integrate into the host cell genome  
20 and express viral genes stably and efficiently have made them attractive candidates for the transfer of foreign genes into mammalian cells (Ridgeway, 1988; Nicolas and Rubenstein, 1988; Baichwal and Sugden, 1986; Temin, 1986). The first viruses used as vectors were DNA viruses including the papovaviruses (simian virus 40, bovine papilloma virus, and polyoma) (Ridgeway, 1988; Baichwal and Sugden, 1986) and adenoviruses (Ridgeway,  
25 1988; Baichwal and Sugden, 1986) and adeno-associated viruses. Retroviruses also are attractive gene transfer vehicles (Nicolas and Rubenstein, 1988; Temin, 1986) as are vaccinia virus (Ridgeway, 1988) and adeno-associated virus (Ridgeway, 1988). Such vectors may be used to (i) transform cell lines *in vitro* for the purpose of expressing  
30 proteins of interest or (ii) to transform cells *in vitro* or *in vivo* to provide therapeutic polypeptides in a gene therapy scenario.

In some embodiments, the vector is HSV. Because HSV is neurotropic, it has generated considerable interest in treating nervous system disorders. Since insulin-secreting pancreatic  $\beta$ -cells share many features with neurons, HSV may be useful for 5 delivering genes to  $\beta$ -cells and for gene therapy of diabetes. Moreover, the ability of HSV to establish latent infections in non-dividing neuronal cells without integrating into the host cell chromosome or otherwise altering the host cell's metabolism, along with the existence of a promoter that is active during latency. And though much attention has focused on the neurotropic applications of HSV, this vector also can be exploited for 10 other tissues.

Another factor that makes HSV an attractive vector is the size and organization of the genome. Because HSV is large, incorporation of multiple genes or expression cassettes is less problematic than in other smaller viral systems. In addition, the 15 availability of different viral control sequences with varying performance (temporal, strength, etc.) makes it possible to control expression to a greater extent than in other systems. It also is an advantage that the virus has relatively few spliced messages, further easing genetic manipulations.

20 HSV also is relatively easy to manipulate and can be grown to high titers. Thus, delivery is less of a problem, both in terms of volumes needed to attain sufficient MOI and in a lessened need for repeat dosings.

#### F. Encoded Proteins

25 Once the entire coding sequence of a marker-associated gene has been determined, the gene can be inserted into an appropriate expression system. The gene can be expressed in any number of different recombinant DNA expression systems to generate large amounts of the polypeptide product, which can then be purified and used to vaccinate animals to 30 generate antisera with which further studies may be conducted.

Examples of expression systems known to the skilled practitioner in the art include bacteria such as *E. coli*, yeast such as *Saccharomyces cerevisiae* and *Pichia pastoris*, baculovirus, and mammalian expression systems such as in COS or CHO cells. In one embodiment, polypeptides are expressed in *E. coli* and in baculovirus expression systems. A complete gene can be expressed or, alternatively, fragments of the gene encoding portions of polypeptide can be produced.

In one embodiment, the gene sequence encoding the polypeptide is analyzed to detect putative transmembrane sequences. Such sequences are typically very hydrophobic and are readily detected by the use of standard sequence analysis software, such as MacVector (IBI, New Haven, CT). The presence of transmembrane sequences is often deleterious when a recombinant protein is synthesized in many expression systems, especially *E. coli*, as it leads to the production of insoluble aggregates that are difficult to renature into the native conformation of the protein. Deletion of transmembrane sequences typically does not significantly alter the conformation of the remaining protein structure.

Moreover, transmembrane sequences, being by definition embedded within a membrane, are inaccessible. Therefore, antibodies to these sequences will not prove useful for *in vivo* or *in situ* studies. Deletion of transmembrane-encoding sequences from the genes used for expression can be achieved by standard techniques. For example, fortuitously-placed restriction enzyme sites can be used to excise the desired gene fragment, or PCR-type amplification can be used to amplify only the desired part of the gene. The skilled practitioner will realize that such changes must be designed so as not to change the translational reading frame for downstream portions of the protein-encoding sequence.

In one embodiment, computer sequence analysis is used to determine the location of the predicted major antigenic determinant epitopes of the polypeptide. Software capable of carrying out this analysis is readily available commercially, for example MacVector (IBI, New Haven, CT). The software typically uses standard algorithms such as the

Kyte/Doolittle or Hopp/Woods methods for locating hydrophilic sequences which are characteristically found on the surface of proteins and are, therefore, likely to act as antigenic determinants.

5       Once this analysis is made, polypeptides can be prepared that contain at least the essential features of the antigenic determinant and that can be employed in the generation of antisera against the polypeptide. Minigenes or gene fusions encoding these determinants can be constructed and inserted into expression vectors by standard methods, for example, using PCR methodology.

10      The gene or gene fragment encoding a polypeptide can be inserted into an expression vector by standard subcloning techniques. In one embodiment, an *E. coli* expression vector is used that produces the recombinant polypeptide as a fusion protein, allowing rapid affinity purification of the protein. Examples of such fusion protein  
15     expression systems are the glutathione S-transferase system (Pharmacia, Piscataway, NJ), the maltose binding protein system (NEB, Beverly, MA), the FLAG system (IBI, New Haven, CT), and the 6xHis system (Qiagen, Chatsworth, CA).

20      Some of these systems produce recombinant polypeptides bearing only a small number of additional amino acids, which are unlikely to affect the antigenic ability of the recombinant polypeptide. For example, both the FLAG system and the 6xHis system add only short sequences, both of that are known to be poorly antigenic and which do not adversely affect folding of the polypeptide to its native conformation. Other fusion systems produce polypeptide where it is desirable to excise the fusion partner from the desired  
25     polypeptide. In one embodiment, the fusion partner is linked to the recombinant polypeptide by a peptide sequence containing a specific recognition sequence for a protease. Examples of suitable sequences are those recognized by the Tobacco Etch Virus protease (Life Technologies, Gaithersburg, MD) or Factor Xa (New England Biolabs, Beverly, MA).

30

Recombinant bacterial cells, for example *E. coli*, are grown in any of a number of suitable media, for example LB, and the expression of the recombinant polypeptide induced by adding IPTG to the media or switching incubation to a higher temperature. After culturing the bacteria for a further period of between 2 and 24 hours, the cells are collected  
5 by centrifugation and washed to remove residual media. The bacterial cells are then lysed, for example, by disruption in a cell homogenizer and centrifuged to separate the dense inclusion bodies and cell membranes from the soluble cell components. This centrifugation can be performed under conditions whereby the dense inclusion bodies are selectively enriched by incorporation of sugars such as sucrose into the buffer and centrifugation at a  
10 selective speed.

In another embodiment, the expression system used is one driven by the baculovirus polyhedron promoter. The gene encoding the polypeptide can be manipulated by standard techniques in order to facilitate cloning into the baculovirus vector. One baculovirus vector  
15 is the pBlueBac vector (Invitrogen, Sorrento, CA). The vector carrying the gene for the polypeptide is transfected into *Spodoptera frugiperda* (Sf9) cells by standard protocols, and the cells are cultured and processed to produce the recombinant antigen. See Summers *et al.*, A MANUAL OF METHODS FOR BACULOVIRUS VECTORS AND INSECT CELL CULTURE PROCEDURES, Texas Agricultural Experimental Station.  
20

As an alternative to recombinant polypeptides, synthetic peptides corresponding to the antigenic determinants can be prepared. Such peptides are at least six amino acid residues long, and may contain up to approximately 35 residues, which is the approximate upper length limit of automated peptide synthesis machines, such as those available from  
25 Applied Biosystems (Foster City, CA). Use of such small peptides for vaccination typically requires conjugation of the peptide to an immunogenic carrier protein such as hepatitis B surface antigen, keyhole limpet hemocyanin or bovine serum albumin. Methods for performing this conjugation are well known in the art.

In one embodiment, amino acid sequence variants of the polypeptide can be prepared. These may, for instance, be minor sequence variants of the polypeptide that arise due to natural variation within the population or they may be homologues found in other species. They also may be sequences that do not occur naturally but that are sufficiently similar that they function similarly and/or elicit an immune response that cross-reacts with natural forms of the polypeptide. Sequence variants can be prepared by standard methods of site-directed mutagenesis such as those described below in the following section.

Amino acid sequence variants of the polypeptide can be substitutional, insertional or deletion variants. Deletion variants lack one or more residues of the native protein which are not essential for function or immunogenic activity, and are exemplified by the variants lacking a transmembrane sequence described above. Another common type of deletion variant is one lacking secretory signal sequences or signal sequences directing a protein to bind to a particular part of a cell. An example of the latter sequence is the SH2 domain, which induces protein binding to phosphotyrosine residues.

Substitutional variants typically contain the exchange of one amino acid for another at one or more sites within the protein, and may be designed to modulate one or more properties of the polypeptide such as stability against proteolytic cleavage. Substitutions preferably are conservative, that is, one amino acid is replaced with one of similar shape and charge. Conservative substitutions are well known in the art and include, for example, the changes of: alanine to serine; arginine to lysine; asparagine to glutamine or histidine; aspartate to glutamate; cysteine to serine; glutamine to asparagine; glutamate to aspartate; glycine to proline; histidine to asparagine or glutamine; isoleucine to leucine or valine; leucine to valine or isoleucine; lysine to arginine; methionine to leucine or isoleucine; phenylalanine to tyrosine, leucine or methionine; serine to threonine; threonine to serine; tryptophan to tyrosine; tyrosine to tryptophan or phenylalanine; and valine to isoleucine or leucine.

Insertional variants include fusion proteins such as those used to allow rapid purification of the polypeptide and also can include hybrid proteins containing sequences from other proteins and polypeptides which are homologues of the polypeptide. For example, an insertional variant could include portions of the amino acid sequence of the 5 polypeptide from one species, together with portions of the homologous polypeptide from another species. Other insertional variants can include those in which additional amino acids are introduced within the coding sequence of the polypeptide. These typically are smaller insertions than the fusion proteins described above and are introduced, for example, into a protease cleavage site.

10

In one embodiment, major antigenic determinants of the polypeptide are identified by an empirical approach in which portions of the gene encoding the polypeptide are expressed in a recombinant host, and the resulting proteins tested for their ability to elicit an immune response. For example, PCR can be used to prepare a range of cDNAs encoding 15 peptides lacking successively longer fragments of the C-terminus of the protein. The immunoprotective activity of each of these peptides then identifies those fragments or domains of the polypeptide that are essential for this activity. Further experiments in which only a small number of amino acids are removed at each iteration then allows the location of the antigenic determinants of the polypeptide.

20

Another embodiment for the preparation of the polypeptides according to the invention is the use of peptide mimetics. Mimetics are peptide-containing molecules that mimic elements of protein secondary structure. See, for example, Johnson *et al.*, "Peptide Turn Mimetics" in *BIOTECHNOLOGY AND PHARMACY*, Pezzuto *et al.*, Eds., Chapman and Hall, New York (1993). The underlying rationale behind the use of peptide mimetics is 25 that the peptide backbone of proteins exists chiefly to orient amino acid side chains in such a way as to facilitate molecular interactions, such as those of antibody and antigen. A peptide mimetic is expected to permit molecular interactions similar to the natural molecule.

30

Successful applications of the peptide mimetic concept have thus far focused on mimetics of  $\beta$ -turns within proteins, which are known to be highly antigenic. Likely  $\beta$ -turn structure within an polypeptide can be predicted by computer-based algorithms as discussed above. Once the component amino acids of the turn are determined, peptide mimetics can  
5 be constructed to achieve a similar spatial orientation of the essential elements of the amino acid side chains.

Modification and changes may be made in the structure of a gene and still obtain a functional molecule that encodes a protein or polypeptide with desirable characteristics. The  
10 following is a discussion based upon changing the amino acids of a protein to create an equivalent, or even an improved, second-generation molecule. The amino acid changes may be achieved by changing the codons of the DNA sequence, according to the following data.

For example, certain amino acids may be substituted for other amino acids in a  
15 protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites on substrate molecules. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid substitutions can be made in a protein sequence, and its underlying DNA coding sequence, and nevertheless obtain a  
20 protein with like properties. It is thus contemplated by the inventors that various changes may be made in the DNA sequences of genes without appreciable loss of their biological utility or activity.

In making such changes, the hydropathic index of amino acids may be considered.  
25 The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte & Doolittle, 1982).

**TABLE 3**

Amino Acids			Codons
Alanine	Ala	A	GCA GCC GCG GCU
Cysteine	Cys	C	UGC UGU
Aspartic acid	Asp	D	GAC GAU
Glutamic acid	Glu	E	GAA GAG
Phenylalanine	Phe	F	UUC UUU
Glycine	Gly	G	GGA GGC GGG GGU
Histidine	His	H	CAC CAU
Isoleucine	Ile	I	AUA AUC AUU
Lysine	Lys	K	AAA AAG
Leucine	Leu	L	UUA UUG CUA CUC CUG CUU
Methionine	Met	M	AUG
Asparagine	Asn	N	AAC AAU
Proline	Pro	P	CCA CCC CCG CCU
Glutamine	Gln	Q	CAA CAG
Arginine	Arg	R	AGA AGG CGA CGC CGG CGU
Serine	Ser	S	AGC AGU UCA UCC UCG UCU
Threonine	Thr	T	ACA ACC ACG ACU
Valine	Val	V	GUU GUC GUG GUU
Tryptophan	Trp	W	UGG
Tyrosine	Tyr	Y	UAC UAU

It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics (Kyte & Doolittle, 1982), these are: Isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, *i.e.*, still obtain a biological functionally equivalent protein. In making such changes, the substitution of amino acids whose hydropathic indices are within  $\pm 2$  is preferred, those which are within  $\pm 1$  are particularly preferred, and those within  $\pm 0.5$  are even more particularly preferred.

It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U.S. Patent 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein.

As detailed in U.S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate ( $+3.0 \pm 1$ ); glutamate ( $+3.0 \pm 1$ ); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline ( $-0.5 \pm 1$ ); alanine (-0.5); histidine -0.5); cysteine (-1.0);

methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent and immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within  $\pm 2$  is preferred, those that are within  $\pm 1$  are particularly preferred, and those within  $\pm 0.5$  are even more particularly preferred.

As outlined above, amino acid substitutions are generally based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions that take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

#### G. Site-Specific Mutagenesis

Site-specific mutagenesis is a technique useful in the preparation of individual peptides, or biologically functional equivalent proteins or peptides, through specific mutagenesis of the underlying DNA. The technique further provides a ready ability to prepare and test sequence variants, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered.

In general, the technique of site-specific mutagenesis is well known in the art. As will be appreciated, the technique typically employs a bacteriophage vector that exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage vectors are commercially available and their use is generally well known to those skilled in the art. Double stranded plasmids are also routinely employed in site directed mutagenesis, which eliminates the step of transferring the gene of interest from a phage to a plasmid.

In general, site-directed mutagenesis is performed by first obtaining a single-stranded vector, or melting of two strands of a double stranded vector which includes within its sequence a DNA sequence encoding the desired protein. An oligonucleotide primer bearing the desired mutated sequence is synthetically prepared. This primer is then annealed with the single-stranded DNA preparation, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as *E. coli* cells, and clones are selected that include recombinant vectors bearing the mutated sequence arrangement.

20

The preparation of sequence variants of the selected gene using site-directed mutagenesis is provided as a means of producing potentially useful species and is not meant to be limiting, as there are other ways in which sequence variants of genes may be obtained. For example, recombinant vectors encoding the desired gene may be treated 25 with mutagenic agents, such as hydroxylamine, to obtain sequence variants.

## H. Expression and Purification of Encoded Proteins

### 1. Expression of Proteins from Cloned cDNAs

The cDNA species specified in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ 30 ID NO:7, and HNF1 $\alpha$  can be expressed as encoded peptides or proteins. In other

embodiments cDNA species specified in SEQ ID NO:78, SEQ ID NO:34, SEQ ID NO:36, SEQ ID NO:38, SEQ ID NO:40, SEQ ID NO:42, SEQ ID NO:44, SEQ ID NO:46, SEQ ID NO:48, SEQ ID NO:50, SEQ ID NO:52, SEQ ID NO:54, and HNF4 $\alpha$  can be expressed as encoded peptides or proteins. The DNA species specified in SEQ ID NO:128 and HNF1 $\beta$

5 can be expressed as encoded peptides or proteins. The engineering of DNA segment(s) for expression in a prokaryotic or eukaryotic system may be performed by techniques generally known to those of skill in recombinant expression. It is believed that virtually any expression system may be employed in the expression of the claimed nucleic acid sequences.

10

Both cDNA and genomic sequences are suitable for eukaryotic expression, as the host cell will generally process the genomic transcripts to yield functional mRNA for translation into protein. Generally speaking, it may be more convenient to employ as the recombinant gene a cDNA version of the gene. It is believed that the use of a cDNA version will provide advantages in that the size of the gene will generally be much smaller and more readily employed to transfect the targeted cell than will a genomic gene, which will typically be up to an order of magnitude larger than the cDNA gene. However, the inventor does not exclude the possibility of employing a genomic version of a particular gene where desired.

15  
20

As used herein, the terms "engineered" and "recombinant" cells are intended to refer to a cell into which an exogenous DNA segment or gene, such as a cDNA or gene has been introduced. Therefore, engineered cells are distinguishable from naturally occurring cells which do not contain a recombinantly introduced exogenous DNA segment or gene. Engineered cells are thus cells having a gene or genes introduced through the hand of man. Recombinant cells include those having an introduced cDNA or genomic DNA, and also include genes positioned adjacent to a promoter not naturally associated with the particular introduced gene.

To express a recombinant encoded protein or peptide, whether mutant or wild-type, in accordance with the present invention one would prepare an expression vector that comprises one of the claimed isolated nucleic acids under the control of one or more promoters. To bring a coding sequence "under the control of" a promoter, one positions  
5 the 5' end of the translational initiation site of the reading frame generally between about 1 and 50 nucleotides "downstream" of (*i.e.*, 3' of) the chosen promoter. The "upstream" promoter stimulates transcription of the inserted DNA and promotes expression of the encoded recombinant protein. This is the meaning of "recombinant expression" in the context used here.

10

Many standard techniques are available to construct expression vectors containing the appropriate nucleic acids and transcriptional/translational control sequences in order to achieve protein or peptide expression in a variety of host-expression systems. Cell types available for expression include, but are not limited to, bacteria, such as *E. coli* and  
15 *B. subtilis* transformed with recombinant phage DNA, plasmid DNA or cosmid DNA expression vectors.

20

Certain examples of prokaryotic hosts are *E. coli* strain RR1, *E. coli* LE392, *E. coli* B, *E. coli*  $\chi$  1776 (ATCC No. 31537) as well as *E. coli* W3110 (F-, lambda-, prototrophic, ATCC No. 273325); bacilli such as *Bacillus subtilis*; and other enterobacteriaceae such as *Salmonella typhimurium*, *Serratia marcescens*, and various *Pseudomonas* species.

25

In general, plasmid vectors containing replicon and control sequences that are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marking sequences that are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is often transformed using pBR322, a plasmid derived from an *E. coli* species. Plasmid pBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR322 plasmid, or other microbial

plasmid or phage must also contain, or be modified to contain, promoters that can be used by the microbial organism for expression of its own proteins.

In addition, phage vectors containing replicon and control sequences that are  
5 compatible with the host microorganism can be used as transforming vectors in connection with these hosts. For example, the phage lambda GEM<sup>TM</sup>-11 may be utilized in making a recombinant phage vector that can be used to transform host cells, such as *E. coli* LE392.

10 Further useful vectors include pIN vectors (Inouye *et al.*, 1985); and pGEX vectors, for use in generating glutathione S-transferase (GST) soluble fusion proteins for later purification and separation or cleavage. Other suitable fusion proteins are those with β-galactosidase, ubiquitin, or the like.

15 Promoters that are most commonly used in recombinant DNA construction include the β-lactamase (penicillinase), lactose and tryptophan (trp) promoter systems. While these are the most commonly used, other microbial promoters have been discovered and utilized, and details concerning their nucleotide sequences have been published, enabling those of skill in the art to ligate them functionally with plasmid  
20 vectors.

For expression in *Saccharomyces*, the plasmid YRp7, for example, is commonly used (Stinchcomb *et al.*, 1979; Kingsman *et al.*, 1979; Tschemper *et al.*, 1980). This plasmid contains the *trp1* gene, which provides a selection marker for a mutant strain of  
25 yeast lacking the ability to grow in tryptophan, for example ATCC No. 44076 or PEP4-1 (Jones, 1977). The presence of the *trp1* lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoting sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase (Hitzeman *et al.*, 1980) or other glycolytic enzymes (Hess *et al.*, 1968; Holland *et al.*, 1978), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. In constructing suitable expression plasmids, the termination sequences associated with these genes are also ligated into the expression vector 3' of the sequence desired to be expressed to provide polyadenylation of the mRNA and termination.

10

Other suitable promoters, which have the additional advantage of transcription controlled by growth conditions, include the promoter region for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the aforementioned glyceraldehyde-3-phosphate dehydrogenase, and  
15 enzymes responsible for maltose and galactose utilization.

In addition to micro-organisms, cultures of cells derived from multicellular organisms may also be used as hosts. In principle, any such cell culture is workable, whether from vertebrate or invertebrate culture. In addition to mammalian cells, these  
20 include insect cell systems infected with recombinant virus expression vectors (*e.g.*, baculovirus); and plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing one or more coding sequences.

25

In a useful insect system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. The isolated nucleic acid coding sequences are cloned into non-essential regions (for example the polyhedron gene) of the virus and placed under control  
30 of an AcNPV promoter (for example, the polyhedron promoter). Successful insertion of

the coding sequences results in the inactivation of the polyhedron gene and production of non-occluded recombinant virus (*i.e.*, virus lacking the proteinaceous coat coded for by the polyhedron gene). These recombinant viruses are then used to infect *Spodoptera frugiperda* cells in which the inserted gene is expressed (*e.g.*, U.S. Patent No. 4,215,051).

5

Examples of useful mammalian host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, WI38, BHK, COS-7, 293, HepG2, NIH3T3, RIN and MDCK cell lines. In addition, a host cell may be chosen that modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion 10 desired. Such modifications (*e.g.*, glycosylation) and processing (*e.g.*, cleavage) of protein products may be important for the function of the encoded protein.

Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. Appropriate cell lines or host 15 systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. Expression vectors for use in mammalian cells ordinarily include an origin of replication (as necessary), a promoter located in front of the gene to be expressed, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation site, and transcriptional terminator sequences. The origin of replication 20 may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral (*e.g.*, Polyoma, Adeno, VSV, BPV) source, or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

25 The promoters may be derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the adenovirus late promoter; the vaccinia virus 7.5K promoter). Further, it is also possible, and may be desirable, to utilize promoter or control sequences normally associated with the desired gene sequence, provided such control sequences are compatible with the host cell 30 systems.

A number of viral based expression systems may be utilized, for example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40 (SV40). The early and late promoters of SV40 virus are useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication. Smaller or larger SV40 fragments may also be used, provided there is included the approximately 250 bp sequence extending from the *Hin*DIII site toward the *Bgl*II site located in the viral origin of replication.

In cases where an adenovirus is used as an expression vector, the coding sequences may be ligated to an adenovirus transcription/translation control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region E1 or E3) will result in a recombinant virus that is viable and capable of expressing proteins in infected hosts.

Specific initiation signals may also be required for efficient translation of the claimed isolated nucleic acid coding sequences. These signals include the ATG initiation codon and adjacent sequences. Exogenous translational control signals, including the ATG initiation codon, may additionally need to be provided. One of ordinary skill in the art would readily be capable of determining this need and providing the necessary signals. It is well known that the initiation codon must be in-frame (or in-phase) with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements or transcription terminators (Bittner *et al.*, 1987).

In eukaryotic expression, one will also typically desire to incorporate into the transcriptional unit an appropriate polyadenylation site (*e.g.*, 5'-AATAAA-3') if one was

not contained within the original cloned segment. Typically, the poly A addition site is placed about 30 to 2000 nucleotides "downstream" of the termination site of the protein at a position prior to transcription termination.

5       For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines that stably express constructs encoding proteins may be engineered. Rather than using expression vectors that contain viral origins of replication, host cells can be transformed with vectors controlled by appropriate expression control elements (*e.g.*, promoter, enhancer, sequences, transcription terminators, polyadenylation  
10 sites, *etc.*), and a selectable marker. Following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched medium, and then are switched to a selective medium. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci, which in turn can be cloned and expanded into  
15 cell lines.

A number of selection systems may be used, including, but not limited, to the herpes simplex virus thymidine kinase (Wigler *et al.*, 1977), hypoxanthine-guanine phosphoribosyltransferase (Szybalska *et al.*, 1962) and adenine phosphoribosyltransferase  
20 genes (Lowy *et al.*, 1980), in *tk*, *hgprt* or *aprt* cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for *dhfr*, which confers resistance to methotrexate (Wigler *et al.*, 1980; O'Hare *et al.*, 1981); *gpt*, which confers resistance to mycophenolic acid (Mulligan *et al.*, 1981); *neo*, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin *et al.*, 1981); and *hygro*, which confers  
25 resistance to hygromycin.

It is contemplated that the isolated nucleic acids of the invention may be "overexpressed", *i.e.*, expressed in increased levels relative to its natural expression in human cells, or even relative to the expression of other proteins in the recombinant host  
30 cell. Such overexpression may be assessed by a variety of methods, including radio-

labeling and/or protein purification. However, simple and direct methods are preferred, for example, those involving SDS/PAGE and protein staining or western blotting, followed by quantitative analyses, such as densitometric scanning of the resultant gel or blot. A specific increase in the level of the recombinant protein or peptide in comparison 5 to the level in natural human cells is indicative of overexpression, as is a relative abundance of the specific protein in relation to the other proteins produced by the host cell and, *e.g.*, visible on a gel.

## 2. *Purification of Expressed Proteins*

10 Further aspects of the present invention concern the purification, and in particular embodiments, the substantial purification, of an encoded protein or peptide. The term "purified protein or peptide" as used herein, is intended to refer to a composition, isolatable from other components, wherein the protein or peptide is purified to any degree relative to its naturally-obtainable state, *i.e.*, in this case, relative to its purity within a 15 hepatocyte or  $\beta$ -cell extract. A purified protein or peptide therefore also refers to a protein or peptide, free from the environment in which it may naturally occur.

20 Generally, "purified" will refer to a protein or peptide composition that has been subjected to fractionation to remove various other components, and which composition substantially retains its expressed biological activity. Where the term "substantially purified" is used, this designation will refer to a composition in which the protein or peptide forms the major component of the composition, such as constituting about 50% or more of the proteins in the composition.

25 Various methods for quantifying the degree of purification of the protein or peptide will be known to those of skill in the art in light of the present disclosure. These include, for example, determining the specific activity of an active fraction, or assessing the number of polypeptides within a fraction by SDS/PAGE analysis. A preferred method for assessing the purity of a fraction is to calculate the specific activity of the 30 fraction, to compare it to the specific activity of the initial extract, and to thus calculate

the degree of purity, herein assessed by a "-fold purification number". The actual units used to represent the amount of activity will, of course, be dependent upon the particular assay technique chosen to follow the purification and whether or not the expressed protein or peptide exhibits a detectable activity.

5

Various techniques suitable for use in protein purification will be well known to those of skill in the art. These include, for example, precipitation with ammonium sulphate, polyethylene glycol, antibodies and the like or by heat denaturation, followed by centrifugation; chromatography steps such as ion exchange, gel filtration, reverse phase, hydroxylapatite and affinity chromatography; isoelectric focusing; gel electrophoresis; and combinations of such and other techniques. As is generally known in the art, it is believed that the order of conducting the various purification steps may be changed, or that certain steps may be omitted, and still result in a suitable method for the preparation of a substantially purified protein or peptide.

10  
15

There is no general requirement that the protein or peptide always be provided in their most purified state. Indeed, it is contemplated that less substantially purified products will have utility in certain embodiments. Partial purification may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same general purification scheme. For example, it is appreciated that a cation-exchange column chromatography performed utilizing an HPLC apparatus will generally result in a greater -fold purification than the same technique utilizing a low pressure chromatography system. Methods exhibiting a lower degree of relative purification may have advantages in total recovery of protein product, or in maintaining the activity of an expressed protein.

It is known that the migration of a polypeptide can vary, sometimes significantly, with different conditions of SDS/PAGE (Capaldi *et al.*, *Biochem. Biophys. Res. Comm.*, 76:425, 1977). It will therefore be appreciated that under differing electrophoresis

conditions, the apparent molecular weights of purified or partially purified expression products may vary.

**I. Preparation of Antibodies Specific for Encoded Proteins**

5

*Antibody Generation*

For some embodiments, it will be desired to produce antibodies that bind with high specificity to the protein product(s) of an isolated nucleic acid selected from the group comprising SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7 or any other mutant of HNF1 $\alpha$ , SEQ ID NO:78, SEQ ID NO:34, SEQ ID NO:36, SEQ ID NO:38, SEQ ID NO:40, SEQ ID NO:42, SEQ ID NO:44, SEQ ID NO:46, SEQ ID NO:48, SEQ ID NO:50, SEQ ID NO:52, SEQ ID NO:54, or any other mutant of HNF4 $\alpha$ , SEQ ID NO:128 (HNF1 $\beta$ ) or any mutant of HNF1 $\beta$ . Means for preparing and characterizing antibodies are well known in the art (See, e.g., Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988; incorporated herein by reference).

Methods for generating polyclonal antibodies are well known in the art. Briefly, a polyclonal antibody is prepared by immunizing an animal with an antigenic composition and collecting antisera from that immunized animal. A wide range of animal species can be used for the production of antisera. Typically the animal used for production of antisera is a rabbit, a mouse, a rat, a hamster, a guinea pig or a goat. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

As is well known in the art, a given composition may vary in its immunogenicity. It is often necessary therefore to boost the host immune system, as may be achieved by coupling a peptide or polypeptide immunogen to a carrier. Exemplary and preferred carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Other albumins such as ovalbumin, mouse serum albumin or rabbit serum albumin can also be used as carriers. Means for conjugating a polypeptide to a carrier protein are well known

in the art and include glutaraldehyde, m-maleimidobenzoyl-N-hydroxysuccinimide ester, carbodiimide and bis-biazotized benzidine.

As is also well known in the art, the immunogenicity of a particular immunogen  
5 composition can be enhanced by the use of non-specific stimulators of the immune response, known as adjuvants. Exemplary and preferred adjuvants include complete Freund's adjuvant (a non-specific stimulator of the immune response containing killed *Mycobacterium tuberculosis*), incomplete Freund's adjuvants and aluminum hydroxide adjuvant.

10

The amount of immunogen composition used in the production of polyclonal antibodies varies upon the nature of the immunogen as well as the animal used for immunization. A variety of routes can be used to administer the immunogen (subcutaneous, intramuscular, intradermal, intravenous and intraperitoneal). The  
15 production of polyclonal antibodies may be monitored by sampling blood of the immunized animal at various points following immunization. A second, booster injection, may also be given. The process of boosting and titering is repeated until a suitable titer is achieved. When a desired level of immunogenicity is obtained, the immunized animal can be bled and the serum isolated and stored, and/or in some cases  
20 the animal can be used to generate MAbs. For production of rabbit polyclonal antibodies, the animal can be bled through an ear vein or alternatively by cardiac puncture. The removed blood is allowed to coagulate and then centrifuged to separate serum components from whole cells and blood clots. The serum may be used as is for various applications or the desired antibody fraction may be purified by well-known methods,  
25 such as affinity chromatography using another antibody or a peptide bound to a solid matrix.

Monoclonal antibodies (MAbs) may be readily prepared through use of well-known techniques, such as those exemplified in U.S. Patent 4,196,265, incorporated  
30 herein by reference. Typically, this technique involves immunizing a suitable animal

with a selected immunogen composition, *e.g.*, a purified or partially purified expressed protein, polypeptide or peptide. The immunizing composition is administered in a manner that effectively stimulates antibody producing cells.

5       The methods for generating monoclonal antibodies (MAbs) generally begin along the same lines as those for preparing polyclonal antibodies. Rodents such as mice and rats are preferred animals, however, the use of rabbit, sheep or frog cells is also possible. The use of rats may provide certain advantages (Goding, 1986, pp. 60-61), but mice are preferred, with the BALB/c mouse being most preferred as this is most routinely used and  
10      generally gives a higher percentage of stable fusions.

The animals are injected with antigen as described above. The antigen may be coupled to carrier molecules such as keyhole limpet hemocyanin if necessary. The antigen would typically be mixed with adjuvant, such as Freund's complete or incomplete  
15      adjuvant. Booster injections with the same antigen would occur at approximately two-week intervals.

Following immunization, somatic cells with the potential for producing antibodies, specifically B lymphocytes (B cells), are selected for use in the MAb  
20      generating protocol. These cells may be obtained from biopsied spleens, tonsils or lymph nodes, or from a peripheral blood sample. Spleen cells and peripheral blood cells are preferred, the former because they are a rich source of antibody-producing cells that are in the dividing plasmablast stage, and the latter because peripheral blood is easily accessible. Often, a panel of animals will have been immunized and the spleen of animal with the  
25      highest antibody titer will be removed and the spleen lymphocytes obtained by homogenizing the spleen with a syringe. Typically, a spleen from an immunized mouse contains approximately  $5 \times 10^7$  to  $2 \times 10^8$  lymphocytes.

30      The antibody-producing B lymphocytes from the immunized animal are then fused with cells of an immortal myeloma cell, generally one of the same species as the

animal that was immunized. Myeloma cell lines suited for use in hybridoma-producing fusion procedures preferably are non-antibody-producing, have high fusion efficiency, and have enzyme deficiencies that render them incapable of growing in certain selective media that support the growth of only the desired fused cells (hybridomas).

5

Any one of a number of myeloma cells may be used, as are known to those of skill in the art (Goding, pp. 65-66, 1986; Campbell, pp. 75-83, 1984). For example, where the immunized animal is a mouse, one may use P3-X63/Ag8, X63-Ag8.653, NS1/1.Ag 4 1, Sp210-Ag14, FO, NSO/U, MPC-11, MPC11-X45-GTG 1.7 and S194/5XX0 Bul; for rats, 10 one may use R210.RCY3, Y3-Ag 1.2.3, IR983F and 4B210; and U-266, GM1500-GRG2, LICR-LON-HMy2 and UC729-6 are all useful in connection with human cell fusions.

One preferred murine myeloma cell is the NS-1 myeloma cell line (also termed P3-NS-1-Ag4-1), which is readily available from the NIGMS Human Genetic Mutant Cell Repository by requesting cell line repository number GM3573. Another mouse myeloma cell line that may be used is the 8-azaguanine-resistant mouse murine myeloma SP2/0 non-producer cell line.

Methods for generating hybrids of antibody-producing spleen or lymph node cells and myeloma cells usually comprise mixing somatic cells with myeloma cells in a 2:1 proportion, though the proportion may vary from about 20:1 to about 1:1, respectively, in the presence of an agent or agents (chemical or electrical) that promote the fusion of cell membranes. Fusion methods using Sendai virus have been described by Kohler and Milstein (1975; 1976), and those using polyethylene glycol (PEG), such as 37% (v/v) 25 PEG, by Gefter *et al.* (1977). The use of electrically induced fusion methods is also appropriate (Goding pp. 71-74, 1986).

Fusion procedures usually produce viable hybrids at low frequencies, about 1 X 10<sup>-6</sup> to 1 X 10<sup>-8</sup>. However, this low frequency does not pose a problem, as the viable, 30 fused hybrids are differentiated from the parental, unfused cells (particularly the unfused

myeloma cells that would normally continue to divide indefinitely) by culturing in a selective medium. The selective medium is generally one that contains an agent that blocks the *de novo* synthesis of nucleotides in the tissue culture media. Exemplary and preferred agents are aminopterin, methotrexate, and azaserine. Aminopterin and  
5 methotrexate block *de novo* synthesis of both purines and pyrimidines, whereas azaserine blocks only purine synthesis. Where aminopterin or methotrexate is used, the media is supplemented with hypoxanthine and thymidine as a source of nucleotides (HAT medium). Where azaserine is used, the media is supplemented with hypoxanthine.

10       The preferred selection medium is HAT. Only cells capable of operating nucleotide salvage pathways are able to survive in HAT medium. The myeloma cells are defective in key enzymes of the salvage pathway, *e.g.*, hypoxanthine phosphoribosyl transferase (HPRT), and thus they cannot survive. The B cells can operate this pathway, but they have a limited life span in culture and generally die within about two weeks.  
15      Therefore, the only cells that can survive in the selective media are those hybrids formed from myeloma and B cells.

20       This culturing provides a population of hybridomas from which specific hybridomas are selected. Typically, selection of hybridomas is performed by culturing the cells by single-clone dilution in microtiter plates, followed by testing the individual clonal supernatants (after about two to three weeks) for the desired reactivity. The assay should be sensitive, simple and rapid, such as radioimmunoassays, enzyme immunoassays, cytotoxicity assays, plaque assays, dot immunobinding assays, and the like.

25       The selected hybridomas would then be serially diluted and cloned into individual antibody-producing cell lines, which can then be propagated indefinitely to provide MAbs. The cell lines may be exploited for MAb production in two basic ways. A sample of the hybridoma can be injected (often into the peritoneal cavity) into a histocompatible animal of the type that was used to provide the somatic and myeloma cells for the original  
30

fusion. The injected animal develops tumors secreting the specific monoclonal antibody produced by the fused cell hybrid. The body fluids of the animal, such as serum or ascites fluid, can then be tapped to provide MAbs in high concentration. The individual cell lines could also be cultured *in vitro*, where the MAbs are naturally secreted into the culture medium from which they can be readily obtained in high concentrations. MAbs produced by either means may be further purified, if desired, using filtration, centrifugation and various chromatographic methods such as HPLC or affinity chromatography.

Large amounts of the monoclonal antibodies of the present invention may also be obtained by multiplying hybridoma cells *in vivo*. Cell clones are injected into mammals that are histocompatible with the parent cells, *e.g.*, syngeneic mice, to cause growth of antibody-producing tumors. Optionally, the animals are primed with a hydrocarbon, especially oils such as pristane (tetramethylpentadecane) prior to injection.

In accordance with the present invention, fragments of the monoclonal antibody of the invention can be obtained from the monoclonal antibody produced as described above, by methods which include digestion with enzymes such as pepsin or papain and/or cleavage of disulfide bonds by chemical reduction. Alternatively, monoclonal antibody fragments encompassed by the present invention can be synthesized using an automated peptide synthesizer, or by expression of full-length gene or of gene fragments in *E. coli*.

The monoclonal conjugates of the present invention are prepared by methods known in the art, *e.g.*, by reacting a monoclonal antibody prepared as described above with, for instance, an enzyme in the presence of a coupling agent such as glutaraldehyde or periodate. Conjugates with fluorescein markers are prepared in the presence of these coupling agents or by reaction with an isothiocyanate. Conjugates with metal chelates are similarly produced. Other moieties to which antibodies may be conjugated include radionuclides such as  $^3\text{H}$ ,  $^{125}\text{I}$ ,  $^{131}\text{I}$ ,  $^{32}\text{P}$ ,  $^{35}\text{S}$ ,  $^{14}\text{C}$ ,  $^{51}\text{Cr}$ ,  $^{36}\text{Cl}$ ,  $^{57}\text{Co}$ ,  $^{58}\text{Co}$ ,  $^{59}\text{Fe}$ ,  $^{75}\text{Se}$ ,  $^{152}\text{Eu}$ , and  $^{99\text{m}}\text{Tc}$ , are other useful labels that can be conjugated to antibodies. Radioactively

labeled monoclonal antibodies of the present invention are produced according to well-known methods in the art. For instance, monoclonal antibodies can be iodinated by contact with sodium or potassium iodide and a chemical oxidizing agent such as sodium hypochlorite, or an enzymatic oxidizing agent, such as lactoperoxidase. Monoclonal  
5 antibodies according to the invention may be labeled with technetium-<sup>99</sup> by ligand exchange process, for example, by reducing pertechnate with stannous solution, chelating the reduced technetium onto a Sephadex column and applying the antibody to this column or by direct labelling techniques, *e.g.*, by incubating pertechnate, a reducing agent such as SNCl<sub>2</sub>, a buffer solution such as sodium-potassium phthalate solution, and the antibody.  
10

It will be appreciated by those of skill in the art that monoclonal or polyclonal antibodies specific for HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  (for proteins that are mutated in MODY3, MODY4, and MODY1) will have utilities in several types of applications. These can include the production of diagnostic kits for use in detecting or diagnosing  
15 MODY3, MODY4, and MODY1 type diabetes. The skilled practitioner will realize that such uses are within the scope of the present invention.

#### J. Immunodetection Assays

20 The immunodetection methods of the present invention have evident utility in the diagnosis of conditions such as MODY3, MODY4, and MODY1 related NIDDM. Here, a biological or clinical sample suspected of containing either the encoded protein or peptide or corresponding antibody is used. However, these embodiments also have applications to non-clinical samples, such as in the titering of antigen or antibody  
25 samples, in the selection of hybridomas, and the like.

In the clinical diagnosis or monitoring of patients with MODY3, MODY4 or MODY1, the detection of an antigen encoded by an HNF1 $\alpha$  nucleic acid, HNF4 $\alpha$  nucleic acid, HNF1 $\beta$  nucleic acid, or a decrease in the levels of such an antigen, in comparison  
30 to the levels in a corresponding biological sample from a normal subject is indicative of a patient with MODY3, MODY4, or MODY1. The basis for such diagnostic methods lies,

in part, with the finding that the nucleic acid HNF1 $\alpha$ , HNF1 $\beta$  and HNF4 $\alpha$  mutants identified in the present invention are responsible for MODY3, MODY4, and MODY1 related diabetes, respectively. Hence, it can be inferred that at least some of these mutations produce elevated levels of encoded proteins, that may also be used as markers  
5 for MODY3, MODY4 or MODY1.

Those of skill in the art are very familiar with differentiating between significant expression of a biomarker, which represents a positive identification, and low level or background expression of a biomarker. Indeed, background expression levels are often  
10 used to form a "cut-off" above which increased staining will be scored as significant or positive. Significant expression may be represented by high levels of antigens in tissues or within body fluids, or alternatively, by a high proportion of cells from within a tissue that each give a positive signal.

15           *1. Immunodetection Methods*

In still further embodiments, the present invention concerns immunodetection methods for binding, purifying, removing, quantifying or otherwise generally detecting biological components. The encoded proteins or peptides of the present invention may be employed to detect antibodies having reactivity therewith, or, alternatively, antibodies prepared in accordance with the present invention, may be employed to detect the encoded proteins or peptides. The steps of various useful immunodetection methods have been described in the scientific literature, such as, e.g., Nakamura *et al.* (1987).

25           In general, the immunobinding methods include obtaining a sample suspected of containing a protein, peptide or antibody, and contacting the sample with an antibody or protein or peptide in accordance with the present invention, as the case may be, under conditions effective to allow the formation of immunocomplexes.

The immunobinding methods include methods for detecting or quantifying the amount of a reactive component in a sample, which methods require the detection or quantitation of any immune complexes formed during the binding process. Here, one  
30

would obtain a sample suspected of containing a HNF1 $\alpha$  or HNF4 $\alpha$  mutant encoded protein, peptide or a corresponding antibody, and contact the sample with an antibody or encoded protein or peptide, as the case may be, and then detect or quantify the amount of immune complexes formed under the specific conditions.

5

In terms of antigen detection, the biological sample analyzed may be any sample that is suspected of containing a HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  antigen, such as a pancreatic  $\beta$ -cell, a homogenized tissue extract, an isolated cell, a cell membrane preparation, separated or purified forms of any of the above protein-containing compositions, or even 10 any biological fluid that comes into contact with diabetic tissue, including blood.

Contacting the chosen biological sample with the protein, peptide or antibody under conditions effective and for a period of time sufficient to allow the formation of immune complexes (primary immune complexes) is generally a matter of simply adding

15 the composition to the sample and incubating the mixture for a period of time long enough for the antibodies to form immune complexes with, *i.e.*, to bind to, any antigens present. After this time, the sample-antibody composition, such as a tissue section, ELISA plate, dot blot or western blot, will generally be washed to remove any non-specifically bound antibody species, allowing only those antibodies specifically bound 20 within the primary immune complexes to be detected.

In general, the detection of immunocomplex formation is well known in the art and may be achieved through the application of numerous approaches. These methods are generally based upon the detection of a label or marker, such as any radioactive, 25 fluorescent, biological or enzymatic tags or labels of standard use in the art. U.S. Patents concerning the use of such labels include 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149 and 4,366,241, each incorporated herein by reference. Of course, one may find additional advantages through the use of a secondary binding ligand such as a second antibody or a biotin/avidin ligand binding arrangement, as is known in the art.

30

The encoded protein, peptide or corresponding antibody employed in the detection may itself be linked to a detectable label, wherein one would then simply detect this label, thereby allowing the amount of the primary immune complexes in the composition to be determined.

5

Alternatively, the first added component that becomes bound within the primary immune complexes may be detected by means of a second binding ligand that has binding affinity for the encoded protein, peptide or corresponding antibody. In these cases, the second binding ligand may be linked to a detectable label. The second binding ligand is 10 itself often an antibody, which may thus be termed a "secondary" antibody. The primary immune complexes are contacted with the labeled, secondary binding ligand, or antibody, under conditions effective and for a period of time sufficient to allow the formation of secondary immune complexes. The secondary immune complexes are then generally washed to remove any non-specifically bound labeled secondary antibodies or ligands, 15 and the remaining label in the secondary immune complexes is then detected.

Further methods include the detection of primary immune complexes by a two step approach. A second binding ligand, such as an antibody, that has binding affinity for the encoded protein, peptide or corresponding antibody is used to form secondary 20 immune complexes, as described above. After washing, the secondary immune complexes are contacted with a third binding ligand or antibody that has binding affinity for the second antibody, again under conditions effective and for a period of time sufficient to allow the formation of immune complexes (tertiary immune complexes). The third ligand or antibody is linked to a detectable label, allowing detection of the 25 tertiary immune complexes thus formed. This system may provide for signal amplification if desired.

## 2. *Immunohistochemistry*

The antibodies of the present invention may also be used in conjunction with both 30 fresh-frozen and formalin-fixed, paraffin-embedded tissue blocks prepared for study by

immunohistochemistry (IHC). For example, each tissue block consists of 50 mg of residual "pulverized" diabetic tissue. The method of preparing tissue blocks from these particulate specimens has been successfully used in previous IHC studies of various prognostic factors, and is well known to those of skill in the art (Brown *et al.*, 1990; 5 Abbondanzo *et al.*, 1990; Allred *et al.*, 1990).

Briefly, frozen-sections may be prepared by rehydrating 50 ng of frozen "pulverized" diabetic tissue at room temperature in phosphate buffered saline (PBS) in small plastic capsules; pelleting the particles by centrifugation; resuspending them in a 10 viscous embedding medium (OCT); inverting the capsule and pelleting again by centrifugation; snap-freezing in -70°C isopentane; cutting the plastic capsule and removing the frozen cylinder of tissue; securing the tissue cylinder on a cryostat microtome chuck; and cutting 25-50 serial sections.

15 Permanent-sections may be prepared by a similar method involving rehydration of the 50 mg sample in a plastic microfuge tube; pelleting; resuspending in 10% formalin for 4 hours fixation; washing/pelleting; resuspending in warm 2.5% agar; pelleting; cooling in ice water to harden the agar; removing the tissue/agar block from the tube; infiltrating and embedding the block in paraffin; and cutting up to 50 serial permanent sections.

20

### 3. ELISA

As noted, it is contemplated that the encoded proteins or peptides of the invention will find utility as immunogens, *e.g.*, in connection with vaccine development, in immunohistochemistry and in ELISA assays. One evident utility of the encoded antigens 25 and corresponding antibodies is in immunoassays for the detection of HNF1 $\alpha$ , HNF1 $\beta$  and HNF4 $\alpha$ , mutant proteins, as needed in diagnosis and prognostic monitoring of MODY.

30 Immunoassays, in their most simple and direct sense, are binding assays. Certain preferred immunoassays are the various types of enzyme linked immunosorbent assays

(ELISA) and radioimmunoassays (RIA) known in the art. Immunohistochemical detection using tissue sections is also particularly useful. However, it will be readily appreciated that detection is not limited to such techniques, and western blotting, dot blotting, FACS analyses, and the like may also be used.

5

In one exemplary ELISA, antibodies binding to the encoded proteins of the invention are immobilized onto a selected surface exhibiting protein affinity, such as a well in a polystyrene microtiter plate. Then, a test composition suspected of containing the HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  mutant, such as a clinical sample, is added to the wells.

10 After binding and washing to remove non-specifically bound immune complexes, the bound antibody may be detected. Detection is generally achieved by the addition of a second antibody specific for the target protein, that is linked to a detectable label. This type of ELISA is a simple "sandwich ELISA". Detection may also be achieved by the addition of a second antibody, followed by the addition of a third antibody that has  
15 binding affinity for the second antibody, with the third antibody being linked to a detectable label.

20 In another exemplary ELISA, the samples suspected of containing the mutant HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  antigen are immobilized onto the well surface and then contacted with the antibodies of the invention. After binding and washing to remove non-specifically bound immune complexes, the bound antigen is detected. Where the initial antibodies are linked to a detectable label, the immune complexes may be detected directly. Again, the immune complexes may be detected using a second antibody that has  
25 binding affinity for the first antibody, with the second antibody being linked to a detectable label.

Another ELISA in which the proteins or peptides are immobilized, involves the use of antibody competition in the detection. In this ELISA, labeled antibodies are added to the wells, allowed to bind to the mutant HNF1 $\alpha$  protein, mutant HNF1 $\beta$  protein or  
30 mutant HNF4 $\alpha$  protein, and detected by means of their label. The amount of marker

antigen in an unknown sample is then determined by mixing the sample with the labeled antibodies before or during incubation with coated wells. The presence of marker antigen in the sample acts to reduce the amount of antibody available for binding to the well and thus reduces the ultimate signal. This is appropriate for detecting antibodies in an  
5 unknown sample, where the unlabeled antibodies bind to the antigen-coated wells and also reduces the amount of antigen available to bind the labeled antibodies.

Irrespective of the format employed, ELISAs have certain features in common, such as coating, incubating or binding, washing to remove non-specifically bound  
10 species, and detecting the bound immune complexes. These are described as follows:

In coating a plate with either antigen or antibody, one will generally incubate the wells of the plate with a solution of the antigen or antibody, either overnight or for a specified period of hours. The wells of the plate will then be washed to remove  
15 incompletely adsorbed material. Any remaining available surfaces of the wells are then "coated" with a nonspecific protein that is antigenically neutral with regard to the test antisera. These include bovine serum albumin (BSA), casein and solutions of milk powder. The coating of nonspecific adsorption sites on the immobilizing surface reduces the background caused by nonspecific binding of antisera to the surface.  
20

In ELISAs, it is probably more customary to use a secondary or tertiary detection means rather than a direct procedure. Thus, after binding of a protein or antibody to the well, coating with a non-reactive material to reduce background, and washing to remove unbound material, the immobilizing surface is contacted with the control MODY3,  
25 MODY4 or MODY1 and/or clinical or biological sample to be tested under conditions effective to allow immune complex (antigen/antibody) formation. Detection of the immune complex then requires a labeled secondary binding ligand or antibody, or a secondary binding ligand or antibody in conjunction with a labeled tertiary antibody or third binding ligand.

"Under conditions effective to allow immune complex (antigen/antibody) formation" means that the conditions preferably include diluting the antigens and antibodies with solutions such as BSA, bovine gamma globulin (BGG) and phosphate buffered saline (PBS)/Tween™. These added agents also tend to assist in the reduction of nonspecific background.

The "suitable" conditions also mean that the incubation is at a temperature and for a period of time sufficient to allow effective binding. Incubation steps are typically from about 1 to 2 to 4 hours, at temperatures preferably on the order of 25° to 27°C, or may be overnight at about 4°C or so.

Following all incubation steps in an ELISA, the contacted surface is washed so as to remove non-complexed material. A preferred washing procedure includes washing with a solution such as PBS/Tween™, or borate buffer. Following the formation of specific immune complexes between the test sample and the originally bound material, and subsequent washing, the occurrence of even minute amounts of immune complexes may be determined.

To provide a detecting means, the second or third antibody will have an associated label to allow detection. Preferably, this label will be an enzyme that will generate color development upon incubating with an appropriate chromogenic substrate. Thus, for example, one will desire to contact and incubate the first or second immune complex with a urease, glucose oxidase, alkaline phosphatase or hydrogen peroxidase-conjugated antibody for a period of time and under conditions that favor the development of further immune complex formation (*e.g.*, incubation for 2 hours at room temperature in a PBS-containing solution such as PBS-Tween™).

After incubation with the labeled antibody, and subsequent to washing to remove unbound material, the amount of label is quantified, *e.g.*, by incubation with a chromogenic substrate such as urea and bromocresol purple or 2,2'-azido-di-(3-ethyl-

benzthiazoline-6-sulfonic acid [ABTS] and H<sub>2</sub>O<sub>2</sub>, in the case of peroxidase as the enzyme label. Quantitation is then achieved by measuring the degree of color generation, e.g., using a visible spectra spectrophotometer.

5           4.     *Use of Antibodies for Radioimaging*

The antibodies of this invention will be used to quantify and localize the expression of the encoded marker proteins. The antibody, for example, will be labeled by any one of a variety of methods and used to visualize the localized concentration of the cells producing the encoded protein. Such an assay also will reveal the subcellular 10 localization of the protein, which can have diagnostic and therapeutic applications.

In accordance with this invention, the monoclonal antibody or fragment thereof may be labeled by any of several techniques known to the art. The methods of the present invention may also use paramagnetic isotopes for purposes of *in vivo* detection. Elements 15 particularly useful in Magnetic Resonance Imaging ("MRI") include <sup>157</sup>Gd, <sup>55</sup>Mn, <sup>162</sup>Dy, <sup>52</sup>Cr, and <sup>56</sup>Fe.

Administration of the labeled antibody may be local or systemic and accomplished intravenously, intraarterially, via the spinal fluid or the like. 20 Administration may also be intradermal or intracavitory, depending upon the body site under examination. After a sufficient time has lapsed for the monoclonal antibody or fragment thereof to bind with the diseased tissue, for example, 30 minutes to 48 hours, the area of the subject under investigation is examined by routine imaging techniques such as MRI, SPECT, planar scintillation imaging or newly emerging imaging 25 techniques. The exact protocol will necessarily vary depending upon factors specific to the patient, as noted above, and depending upon the body site under examination, method of administration and type of label used; the determination of specific procedures would be routine to the skilled artisan. The distribution of the bound radioactive isotope and its increase or decrease with time is then monitored and recorded. By comparing the results

with data obtained from studies of clinically normal individuals, the presence and extent of the diseased tissue can be determined.

It will be apparent to those of skill in the art that a similar approach may be used  
5 to radio-image the production of the encoded HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  mutant proteins in human patients. The present invention provides methods for the *in vivo* diagnosis of MODY3, MODY4 or MODY1 in a patient. Such methods generally comprise administering to a patient an effective amount of an HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  mutant  
10 specific antibody, to which antibody is conjugated a marker, such as a radioactive isotope or a spin-labeled molecule, that is detectable by non-invasive methods. The antibody-marker conjugate is allowed sufficient time to come into contact with reactive antigens that are present within the tissues of the patient, and the patient is then exposed to a detection device to identify the detectable marker.

15           5.     *Kits*

In still further embodiments, the present invention concerns immunodetection kits for use with the immunodetection methods described above. As the encoded proteins or peptides may be employed to detect antibodies and the corresponding antibodies may be employed to detect encoded proteins or peptides, either or both of such components may  
20 be provided in the kit. The immunodetection kits will thus comprise, in suitable container means, an encoded protein or peptide, or a first antibody that binds to an encoded protein or peptide, and an immunodetection reagent.

25           In certain embodiments, the encoded protein or peptide, or the first antibody that binds to the encoded protein or peptide, may be bound to a solid support, such as a column matrix or well of a microtiter plate.

30           The immunodetection reagents of the kit may take any one of a variety of forms, including those detectable labels that are associated with or linked to the given antibody or antigen, and detectable labels that are associated with or attached to a secondary

binding ligand. Exemplary secondary ligands are those secondary antibodies that have binding affinity for the first antibody or antigen, and secondary antibodies that have binding affinity for a human antibody.

5       Further suitable immunodetection reagents for use in the present kits include the two-component reagent that comprises a secondary antibody that has binding affinity for the first antibody or antigen, along with a third antibody that has binding affinity for the second antibody, the third antibody being linked to a detectable label.

10      The kits may further comprise a suitably aliquoted composition of the encoded protein or polypeptide antigen, whether labeled or unlabeled, as may be used to prepare a standard curve for a detection assay.

15      The kits may contain antibody-label conjugates either in fully conjugated form, in the form of intermediates, or as separate moieties to be conjugated by the user of the kit. The components of the kits may be packaged either in aqueous media or in lyophilized form.

20      The container means of the kits will generally include at least one vial, test tube, flask, bottle, syringe or other container means, into which the antibody or antigen may be placed, and preferably, suitably aliquoted. Where a second or third binding ligand or additional component is provided, the kit will also generally contain a second, third or other additional container into which this ligand or component may be placed. The kits of the present invention will also typically include a means for containing the antibody, 25     antigen, and any other reagent containers in close confinement for commercial sale. Such containers may include injection or blow-molded plastic containers into which the desired vials are retained.

## K. Detection and Quantitation of Nucleic Acid Species

One embodiment of the instant invention comprises a method for identification of HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  mutants in a biological sample by amplifying and detecting nucleic acids corresponding to HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  mutants. The biological sample can be any tissue or fluid in which these mutants might be present. Various embodiments include  $\beta$  and  $\alpha$ -cells of pancreatic islets, bone marrow aspirate, bone marrow biopsy, lymph node aspirate, lymph node biopsy, spleen tissue, fine needle aspirate, skin biopsy or organ tissue biopsy. Other embodiments include samples where the body fluid is peripheral blood, lymph fluid, ascites, serous fluid, pleural effusion, sputum, cerebrospinal fluid, lacrimal fluid, stool or urine.

Nucleic acid used as a template for amplification is isolated from cells contained in the biological sample, according to standard methodologies (Sambrook *et al.*, 1989). The nucleic acid may be genomic DNA or fractionated or whole cell RNA. Where RNA is used, it may be desired to convert the RNA to a complementary DNA. In one embodiment, the RNA is whole cell RNA and is used directly as the template for amplification.

Pairs of primers that selectively hybridize to nucleic acids corresponding to HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  mutants are contacted with the isolated nucleic acid under conditions that permit selective hybridization. Once hybridized, the nucleic acid:primer complex is contacted with one or more enzymes that facilitate template-dependent nucleic acid synthesis. Multiple rounds of amplification, also referred to as "cycles," are conducted until a sufficient amount of amplification product is produced.

Next, the amplification product is detected. In certain applications, the detection may be performed by visual means. Alternatively, the detection may involve indirect identification of the product via chemiluminescence, radioactive scintigraphy or incorporated radiolabel or fluorescent label or even via a system using electrical or thermal impulse signals (Affymax technology; Bellus, 1994).

Following detection, one may compare the results seen in a given patient with a statistically significant reference group of normal patients and MODY or indeed MODY dependent diabetics and non MODY dependent diabetics. In this way, it is possible to  
5 correlate the amount of HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  mutants detected with various clinical states.

1. *Primers*

The term primer, as defined herein, is meant to encompass any nucleic acid that is  
10 capable of priming the synthesis of a nascent nucleic acid in a template-dependent process. Typically, primers are oligonucleotides from ten to twenty base pairs in length, but longer sequences can be employed. Primers may be provided in double-stranded or single-stranded form, although the single-stranded form is preferred.

15 2. *Template Dependent Amplification Methods*

A number of template dependent processes are available to amplify the marker sequences present in a given template sample. One of the best known amplification methods is the polymerase chain reaction (referred to as PCR) which is described in detail in U.S. Patent Nos. 4,683,195, 4,683,202 and 4,800,159, and in Innis *et al.*, 1990, each of  
20 which is incorporated herein by reference in its entirety.

Briefly, in PCR, two primer sequences are prepared that are complementary to regions on opposite complementary strands of the marker sequence. An excess of deoxynucleoside triphosphates are added to a reaction mixture along with a DNA  
25 polymerase, *e.g.*, *Taq* polymerase. If the marker sequence is present in a sample, the primers will bind to the marker and the polymerase will cause the primers to be extended along the marker sequence by adding on nucleotides. By raising and lowering the temperature of the reaction mixture, the extended primers will dissociate from the marker to form reaction products, excess primers will bind to the marker and to the reaction  
30 products and the process is repeated.

A reverse transcriptase PCR amplification procedure may be performed in order to quantify the amount of mRNA amplified. Methods of reverse transcribing RNA into cDNA are well known and described in Sambrook *et al.*, 1989. Alternative methods for 5 reverse transcription utilize thermostable, RNA-dependent DNA polymerases. These methods are described in WO 90/07641 filed December 21, 1990. Polymerase chain reaction methodologies are well known in the art.

Another method for amplification is the ligase chain reaction ("LCR"), disclosed 10 in EPA No. 320 308, incorporated herein by reference in its entirety. In LCR, two complementary probe pairs are prepared, and in the presence of the target sequence, each pair will bind to opposite complementary strands of the target such that they abut. In the presence of a ligase, the two probe pairs will link to form a single unit. By temperature cycling, as in PCR, bound ligated units dissociate from the target and then serve as "target 15 sequences" for ligation of excess probe pairs. U.S. Patent 4,883,750 describes a method similar to LCR for binding probe pairs to a target sequence.

Qbeta Replicase, described in PCT Application No. PCT/US87/00880, may also 20 be used as still another amplification method in the present invention. In this method, a replicative sequence of RNA that has a region complementary to that of a target is added to a sample in the presence of an RNA polymerase. The polymerase will copy the replicative sequence that can then be detected.

An isothermal amplification method, in which restriction endonucleases and 25 ligases are used to achieve the amplification of target molecules that contain nucleotide 5'-[alpha-thio]-triphosphates in one strand of a restriction site may also be useful in the amplification of nucleic acids in the present invention, Walker *et al.*, (1992), incorporated herein by reference in its entirety.

Strand Displacement Amplification (SDA) is another method of carrying out isothermal amplification of nucleic acids which involves multiple rounds of strand displacement and synthesis, *i.e.*, nick translation. A similar method, called Repair Chain Reaction (RCR), involves annealing several probes throughout a region targeted for

5 amplification, followed by a repair reaction in which only two of the four bases are present. The other two bases can be added as biotinylated derivatives for easy detection. A similar approach is used in SDA. Target specific sequences can also be detected using a cyclic probe reaction (CPR). In CPR, a probe having 3' and 5' sequences of non-specific DNA and a middle sequence of specific RNA is hybridized to DNA that is

10 present in a sample. Upon hybridization, the reaction is treated with RNase H, and the products of the probe identified as distinctive products that are released after digestion. The original template is annealed to another cycling probe and the reaction is repeated.

Still another amplification methods described in GB Application No. 2 202 328,  
15 and in PCT Application No. PCT/US89/01025, each of which is incorporated herein by reference in its entirety, may be used in accordance with the present invention. In the former application, "modified" primers are used in a PCR-like, template- and enzyme-dependent synthesis. The primers may be modified by labelling with a capture moiety (*e.g.*, biotin) and/or a detector moiety (*e.g.*, enzyme). In the latter application, an excess  
20 of labeled probes are added to a sample. In the presence of the target sequence, the probe binds and is cleaved catalytically. After cleavage, the target sequence is released intact to be bound by excess probe. Cleavage of the labeled probe signals the presence of the target sequence.

25 Other nucleic acid amplification procedures include transcription-based amplification systems (TAS), including nucleic acid sequence based amplification (NASBA) and 3SR (Kwoh *et al.*, 1989); Gingeras *et al.*, PCT Application WO 88/10315, incorporated herein by reference in their entirety). In NASBA, the nucleic acids can be prepared for amplification by standard phenol/chloroform extraction, heat denaturation of  
30 a clinical sample, treatment with lysis buffer and minispin columns for isolation of DNA

and RNA or guanidinium chloride extraction of RNA. These amplification techniques involve annealing a primer which has target specific sequences. Following polymerization, DNA/RNA hybrids are digested with RNase H while double stranded DNA molecules are heat denatured again. In either case the single stranded DNA is made  
5 fully double stranded by addition of second target specific primer, followed by polymerization. The double-stranded DNA molecules are then multiply transcribed by an RNA polymerase such as T7 or SP6. In an isothermal cyclic reaction, the RNA's are reverse transcribed into single stranded DNA, which is then converted to double stranded DNA, and then transcribed once again with an RNA polymerase such as T7 or SP6. The  
10 resulting products, whether truncated or complete, indicate target specific sequences.

Davey *et al.*, EPA No. 329 822 (incorporated herein by reference in its entirety) disclose a nucleic acid amplification process involving cyclically synthesizing single-stranded RNA ("ssRNA"), ssDNA, and double-stranded DNA (dsDNA), which may be  
15 used in accordance with the present invention. The ssRNA is a template for a first primer oligonucleotide, which is elongated by reverse transcriptase (RNA-dependent DNA polymerase). The RNA is then removed from the resulting DNA:RNA duplex by the action of ribonuclease H (RNase H, an RNase specific for RNA in duplex with either DNA or RNA). The resultant ssDNA is a template for a second primer, which also  
20 includes the sequences of an RNA polymerase promoter (exemplified by T7 RNA polymerase) 5' to its homology to the template. This primer is then extended by DNA polymerase (exemplified by the large "Klenow" fragment of *E. coli* DNA polymerase I), resulting in a double-stranded DNA ("dsDNA") molecule, having a sequence identical to that of the original RNA between the primers and having additionally, at one end, a  
25 promoter sequence. This promoter sequence can be used by the appropriate RNA polymerase to make many RNA copies of the DNA. These copies can then re-enter the cycle leading to very swift amplification. With proper choice of enzymes, this amplification can be done isothermally without addition of enzymes at each cycle. Because of the cyclical nature of this process, the starting sequence can be chosen to be in  
30 the form of either DNA or RNA.

Miller *et al.*, PCT Application WO 89/06700 (incorporated herein by reference in its entirety) disclose a nucleic acid sequence amplification scheme based on the hybridization of a promoter/primer sequence to a target single-stranded DNA ("ssDNA") followed by transcription of many RNA copies of the sequence. This scheme is not cyclic, *i.e.*, new templates are not produced from the resultant RNA transcripts. Other amplification methods include "RACE" and "one-sided PCR" (Frohman, M.A., In: *PCR PROTOCOLS: A GUIDE TO METHODS AND APPLICATIONS*, Academic Press, N.Y., 1990; Ohara *et al.*, 1989; each herein incorporated by reference in their entirety).

10

Methods based on ligation of two (or more) oligonucleotides in the presence of nucleic acid having the sequence of the resulting "di-oligonucleotide", thereby amplifying the di-oligonucleotide, may also be used in the amplification step of the present invention. Wu *et al.*, 1989), incorporated herein by reference in its entirety.

15

### 3. *RNase Protection Assay*

Methods for genetic screening by identifying mutations associated with most genetic diseases such as diabetes must be able to assess large regions of the genome. Once a relevant mutation has been identified in a given patient, other family members and affected individuals can be screened using methods which are targeted to that site. The ability to detect dispersed point mutations is critical for genetic counseling, diagnosis, and early clinical intervention as well as for research into the etiology of cancer and other genetic disorders. The ideal method for genetic screening would quickly, inexpensively, and accurately detect all types of widely dispersed mutations in genomic DNA, cDNA, and RNA samples, depending on the specific situation.

Historically, a number of different methods have been used to detect point mutations, including denaturing gradient gel electrophoresis ("DGGE"), restriction enzyme polymorphism analysis, chemical and enzymatic cleavage methods, and others (Cotton, 1989). The more common procedures currently in use include direct sequencing

of target regions amplified by PCR<sup>TM</sup> and single-strand conformation polymorphism analysis ("SSCP").

Another method of screening for point mutations is based on RNase cleavage of  
5 base pair mismatches in RNA/DNA and RNA/RNA heteroduplexes. As used herein, the term "mismatch" is defined as a region of one or more unpaired or mispaired nucleotides in a double-stranded RNA/RNA, RNA/DNA or DNA/DNA molecule. This definition thus includes mismatches due to insertion/deletion mutations, as well as single and multiple base point mutations. U.S. Patent No. 4,946,773 describes an RNase A  
10 mismatch cleavage assay that involves annealing single-stranded DNA or RNA test samples to an RNA probe, and subsequent treatment of the nucleic acid duplexes with RNase A. After the RNase cleavage reaction, the RNase is inactivated by proteolytic digestion and organic extraction, and the cleavage products are denatured by heating and analyzed by electrophoresis on denaturing polyacrylamide gels. For the detection of  
15 mismatches, the single-stranded products of the RNase A treatment, electrophoretically separated according to size, are compared to similarly treated control duplexes. Samples containing smaller fragments (cleavage products) not seen in the control duplex are scored as +.

20 Currently available RNase mismatch cleavage assays, including those performed according to U.S. Patent No. 4,946,773, require the use of radiolabeled RNA probes. Myers and Maniatis in U.S. Patent No. 4,946,773 describe the detection of base pair mismatches using RNase A. Other investigators have described the use of *E.coli* enzyme, RNase I, in mismatch assays. Because it has broader cleavage specificity than  
25 RNase A, RNase I would be a desirable enzyme to employ in the detection of base pair mismatches if components can be found to decrease the extent of non-specific cleavage and increase the frequency of cleavage of mismatches. The use of RNase I for mismatch detection is described in literature from Promega Biotech. Promega markets a kit containing RNase I that is shown in their literature to cleave three out of four known  
30 mismatches, provided the enzyme level is sufficiently high.

The RNase protection assay as first described by Melton *et al.* (1984) was used to detect and map the ends of specific mRNA targets in solution. The assay relies on being able to easily generate high specific activity radiolabeled RNA probes complementary to the mRNA of interest by *in vitro* transcription. Originally, the templates for *in vitro* transcription were recombinant plasmids containing bacteriophage promoters. The probes are mixed with total cellular RNA samples to permit hybridization to their complementary targets, then the mixture is treated with RNase to degrade excess unhybridized probe. Also, as originally intended, the RNase used is specific for single-stranded RNA, so that hybridized double-stranded probe is protected from degradation. After inactivation and removal of the RNase, the protected probe (which is proportional in amount to the amount of target mRNA that was present) is recovered and analyzed on a polyacrylamide gel.

The RNase Protection assay was adapted for detection of single base mutations by Myers and Maniatis (1985) and by Winter and Perucho (1985). In this type of RNase A mismatch cleavage assay, radiolabeled RNA probes transcribed *in vitro* from wild type sequences, are hybridized to complementary target regions derived from test samples. The test target generally comprises DNA (either genomic DNA or DNA amplified by cloning in plasmids or by PCR<sup>TM</sup>), although RNA targets (endogenous mRNA) have occasionally been used (Gibbs and Caskey, 1987; Winter *et al.*, 1985). If single nucleotide (or greater) sequence differences occur between the hybridized probe and target, the resulting disruption in Watson-Crick hydrogen bonding at that position ("mismatch") can be recognized and cleaved in some cases by single-strand specific ribonuclease. To date, RNase A has been used almost exclusively for cleavage of single-base mismatches, although RNase I has recently been shown as useful also for mismatch cleavage. There are recent descriptions of using the MutS protein and other DNA-repair enzymes for detection of single-base mismatches (Ellis *et al.*, 1994; Lishanski *et al.*, 1994).

By hybridizing each strand of the wild type probe in RNase cleavage mismatch assays separately to the complementary Sense and Antisense strands of the test target, two different complementary mismatches (for example, A-C and G-U or G-T) and therefore two chances for detecting each mutation by separate cleavage events, was provided.

5 Myers *et al.* (1985) used the RNase A cleavage assay to screen 615 bp regions of the human  $\beta$ -globin gene contained in recombinant plasmid targets. By probing with both strands, they were able to detect most, but not all, of the  $\beta$ -globin mutations in their model system. The collection of mutants included examples of all the 12 possible types of mismatches between RNA and DNA: rA/dA, rC/dC, rU/dC, rC/dA, rC/dT, rU/dG,

10 rG/dA, rG/dG, rU/dG, rA/dC, rG/dT, and rA/dG.

Myers *et. al.* (1985) showed that certain types of mismatch were more frequently and more completely cleaved by RNase A than others. For example, the rC/dA, rC/dC, and rC/dT mismatches were cleaved in all cases, while the rG/dA mismatch was only 15 cleaved in 13% of the cases tested and the rG/dT mismatch was almost completely resistant to cleavage. In general, the complement of a difficult-to-detect mismatch was much easier to detect. For example, the refractory rG/dT mismatch generated by probing a G to A mutant target with a wild type sense-strand probe, is complemented by the easily cleaved rC/dA mismatch generated by probing the mutant target with the wild type 20 antisense strand. By probing both target strands, Myers and Maniatis (1986) estimated that at least 50% of all single-base mutations would be detected by the RNase A cleavage assay. These authors stated that approximately one-third of all possible types of single-base substitutions would be detected by using a single probe for just one strand of the target DNA (Myers *et al.*, 1985).

25

In the typical RNase cleavage assays, the separating gels are run under denaturing conditions for analysis of the cleavage products. This requires the RNase to be inactivated by treating the reaction with protease (usually Proteinase K, often in the presence of SDS) to degrade the RNase. This reaction is generally followed by an 30 organic extraction with a phenol/chloroform solution to remove proteins and residual

RNase activity. The organic extraction is then followed by concentration and recovery of the cleavage products by alcohol precipitation (Myers *et al.*, 1985; Winter *et al.*, 1985; Theophilus *et al.*, 1989).

5           4.     *Separation Methods*

Following amplification, it may be desirable to separate the amplification product from the template and the excess primer for the purpose of determining whether specific amplification has occurred. In one embodiment, amplification products are separated by agarose, agarose-acrylamide or polyacrylamide gel electrophoresis using standard 10 methods. See Sambrook *et al.*, 1989.

15           Alternatively, chromatographic techniques may be employed to effect separation. There are many kinds of chromatography which may be used in the present invention: adsorption, partition, ion-exchange and molecular sieve, and many specialized techniques for using them including column, paper, thin-layer and gas chromatography (Freifelder, 1982).

5           5.     *Identification Methods*

20           Amplification products must be visualized in order to confirm amplification of the marker sequences. One typical visualization method involves staining of a gel with ethidium bromide and visualization under UV light. Alternatively, if the amplification products are integrally labeled with radio- or fluorometrically-labeled nucleotides, the amplification products can then be exposed to x-ray film or visualized under the appropriate stimulating spectra, following separation.

25

In one embodiment, visualization is achieved indirectly. Following separation of amplification products, a labeled, nucleic acid probe is brought into contact with the amplified marker sequence. The probe preferably is conjugated to a chromophore but may be radiolabeled. In another embodiment, the probe is conjugated to a binding

partner, such as an antibody or biotin, and the other member of the binding pair carries a detectable moiety.

In one embodiment, detection is by Southern blotting and hybridization with a  
5 labeled probe. The techniques involved in Southern blotting are well known to those of skill in the art and can be found in many standard books on molecular protocols. See Sambrook *et al.*, 1989. Briefly, amplification products are separated by gel electrophoresis. The gel is then contacted with a membrane, such as nitrocellulose, permitting transfer of the nucleic acid and non-covalent binding. Subsequently, the  
10 membrane is incubated with a chromophore-conjugated probe that is capable of hybridizing with a target amplification product. Detection is by exposure of the membrane to x-ray film or ion-emitting detection devices.

One example of the foregoing is described in U.S. Patent No. 5,279,721,  
15 incorporated by reference herein, which discloses an apparatus and method for the automated electrophoresis and transfer of nucleic acids. The apparatus permits electrophoresis and blotting without external manipulation of the gel and is ideally suited to carrying out methods according to the present invention.

20           6.     *Kit Components*

All the essential materials and reagents required for detecting MODY markers in a biological sample may be assembled together in a kit. This generally will comprise pre-selected primers for specific markers. Also included may be enzymes suitable for amplifying nucleic acids including various polymerases (RT, Taq, etc.), deoxynucleotides  
25 and buffers to provide the necessary reaction mixture for amplification.

Such kits generally will comprise, in suitable means, distinct containers for each individual reagent and enzyme as well as for each marker primer pair. Preferred pairs of primers for amplifying nucleic acids are selected to amplify the sequences specified in  
30 SEQ ID NO:3, SEQ ID NO:5, or SEQ ID NO:5, along with the cDNAs for HNF1 $\alpha$  (SEQ

ID NO:1) HNF1 $\beta$  (SEQ ID NO:128) and HNF4 $\alpha$  (SEQ ID NO:78). In other embodiments preferred pairs of primers for amplification are selected to amplify sequences specified in SEQ ID NO:34, SEQ ID NO:36, SEQ ID NO:38, SEQ ID NO:40, SEQ ID NO:42, SEQ ID NO:44, SEQ ID NO:46, SEQ ID NO:48, SEQ ID NO:50, SEQ ID NO:52, SEQ ID NO:54.

In another embodiment, such kits will comprise hybridization probes specific for MODY3, chosen from a group including nucleic acids corresponding to the sequences specified in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, and SEQ ID NO:7, along with the cDNAs for HNF1 $\alpha$  (SEQ ID NO:1). In yet another embodiment such kits will comprise probes specific for MODY 1 chosen from a group including nucleic acids corresponding to the sequences specified in SEQ ID NO:78, SEQ ID NO:34, SEQ ID NO:36, SEQ ID NO:38, SEQ ID NO:40, SEQ ID NO:42, SEQ ID NO:44, SEQ ID NO:46, SEQ ID NO:48, SEQ ID NO:50, SEQ ID NO:52, SEQ ID NO:54, HNF4 $\alpha$ . In still another embodiment such kits will comprise probes specific for MODY4 chosen from a group including nucleic acids corresponding to the sequences specified in SEQ ID NO:128, HNF1 $\beta$  or any of the exons shown in FIG. 27A-FIG. 27I, or Genbank accession numbers U90279-90287 and U96079, incorporated herein by reference.

Such kits generally will comprise, in suitable means, distinct containers for each individual reagent and enzyme as well as for each marker hybridization probe..

#### L. Use of RNA Fingerprinting to Identify MODY3, MODY4, and MODY1 Markers

RNA fingerprinting is a means by which RNAs isolated from many different tissues, cell types or treatment groups can be sampled simultaneously to identify RNAs whose relative abundances vary. Two forms of this technology were developed simultaneously and reported in 1992 as RNA fingerprinting by differential display (Liang and Pardee, 1992; Welsh *et al.*, 1992). (*See also* Liang and Pardee, U.S. patent 5,262,311, incorporated herein by reference in its entirety.) Some of the experiments

described herein were performed similarly to Donahue *et al.*, *J. Biol. Chem.* 269: 8604-8609, 1994.

All forms of RNA fingerprinting by PCR are theoretically similar but differ in  
5 their primer design and application. The most striking difference between differential display and other methods of RNA fingerprinting is that differential display utilizes anchoring primers that hybridize to the poly A tails of mRNAs. As a consequence, the PCR products amplified in differential display are biased towards the 3' untranslated regions of mRNAs.

10

The basic technique of differential display has been described in detail (Liang and Pardee, 1992). Total cell RNA is primed for first strand reverse transcription with an anchoring primer composed of oligo dT and any two of the four deoxynucleosides. The oligo dT primer is extended using a reverse transcriptase, for example, Moloney Murine  
15 Leukemia Virus (MMLV) reverse transcriptase. The synthesis of the second strand is primed with an arbitrarily chosen oligonucleotide, using reduced stringency conditions. Once the double-stranded cDNA has been synthesized, amplification proceeds by standard PCR techniques, utilizing the same primers. The resulting DNA fingerprint is analyzed by gel electrophoresis and ethidium bromide staining or autoradiography. A  
20 side by side comparison of fingerprints obtained from for example tumor *versus* normal tissue samples using the same oligonucleotide primers identifies mRNAs that are differentially expressed.

RNA fingerprinting technology has been demonstrated as being effective in  
25 identifying genes that are differentially expressed in cancer (Liang *et al.*, 1992; Wong *et al.*, 1993; Sager *et al.*, 1993; Mok *et al.*, 1994; Watson *et al.*, 1994; Chen *et al.*, 1995; An *et al.*, 1995). The present invention utilizes the RNA fingerprinting technique to identify genes that are differentially expressed in diabetes.

30 *Design and Theoretical Considerations for Relative Quantitative RT-PCR*

Reverse transcription (RT) of RNA to cDNA followed by relative quantitative PCR (RT-PCR) can be used to determine the relative concentrations of specific mRNA species isolated from MODY3, MODY4, and MODY1 patients. By determining that the concentration of a specific mRNA species varies, it is shown that the gene encoding the specific mRNA species is differentially expressed. This technique can be used to confirm that mRNA transcripts shown to be differentially regulated by RNA fingerprinting are differentially expressed in MODY related diabetes.

In PCR, the number of molecules of the amplified target DNA increase by a factor approaching two with every cycle of the reaction until some reagent becomes limiting. Thereafter, the rate of amplification becomes increasingly diminished until there is no increase in the amplified target between cycles. If a graph is plotted in which the cycle number is on the X axis and the log of the concentration of the amplified target DNA is on the Y axis, a curved line of characteristic shape is formed by connecting the plotted points. Beginning with the first cycle, the slope of the line is positive and constant. This is said to be the linear portion of the curve. After a reagent becomes limiting, the slope of the line begins to decrease and eventually becomes zero. At this point the concentration of the amplified target DNA becomes asymptotic to some fixed value. This is said to be the plateau portion of the curve.

The concentration of the target DNA in the linear portion of the PCR amplification is directly proportional to the starting concentration of the target before the reaction began. By determining the concentration of the amplified products of the target DNA in PCR reactions that have completed the same number of cycles and are in their linear ranges, it is possible to determine the relative concentrations of the specific target sequence in the original DNA mixture. If the DNA mixtures are cDNAs synthesized from RNAs isolated from different tissues or cells, the relative abundances of the specific mRNA from which the target sequence was derived can be determined for the respective tissues or cells. This direct proportionality between the concentration of the PCR products and the relative mRNA abundances is only true in the linear range of the PCR reaction.

The final concentration of the target DNA in the plateau portion of the curve is determined by the availability of reagents in the reaction mix and is independent of the original concentration of target DNA. Therefore, the first condition that must be met  
5 before the relative abundances of a mRNA species can be determined by RT-PCR for a collection of RNA populations is that the concentrations of the amplified PCR products must be sampled when the PCR reactions are in the linear portion of their curves.

The second condition that must be met for an RT-PCR experiment to successfully  
10 determine the relative abundances of a particular mRNA species is that relative concentrations of the amplifiable cDNAs must be normalized to some independent standard. The goal of an RT-PCR experiment is to determine the abundance of a particular mRNA species relative to the average abundance of all mRNA species in the sample. In the experiments described below, mRNAs for  $\beta$ -actin, asparagine synthetase  
15 and lipocortin II were used as external and internal standards to which the relative abundance of other mRNAs are compared.

Most protocols for competitive PCR utilize internal PCR standards that are approximately as abundant as the target. These strategies are effective if the products of  
20 the PCR amplifications are sampled during their linear phases. If the products are sampled when the reactions are approaching the plateau phase, then the less abundant product becomes relatively over represented. Comparisons of relative abundances made for many different RNA samples, such as is the case when examining RNA samples for differential expression, become distorted in such a way as to make differences in relative  
25 abundances of RNAs appear less than they actually are. This is not a significant problem if the internal standard is much more abundant than the target. If the internal standard is more abundant than the target, then direct linear comparisons can be made between RNA samples.

The above discussion describes theoretical considerations for an RT-PCR assay for clinically derived materials. The problems inherent in clinical samples are that they are of variable quantity (making normalization problematic), and that they are of variable quality (necessitating the co-amplification of a reliable internal control, preferably of  
5 larger size than the target). Both of these problems are overcome if the RT-PCR is performed as a relative quantitative RT-PCR with an internal standard in which the internal standard is an amplifiable cDNA fragment that is larger than the target cDNA fragment and in which the abundance of the mRNA encoding the internal standard is roughly 5-100 fold higher than the mRNA encoding the target. This assay measures  
10 relative abundance, not absolute abundance of the respective mRNA species.

Other studies may be performed using a more conventional relative quantitative RT-PCR assay with an external standard protocol. These assays sample the PCR products in the linear portion of their amplification curves. The number of PCR cycles  
15 that are optimal for sampling must be empirically determined for each target cDNA fragment. In addition, the reverse transcriptase products of each RNA population isolated from the various tissue samples must be carefully normalized for equal concentrations of amplifiable cDNAs. This consideration is very important since the assay measures absolute mRNA abundance. Absolute mRNA abundance can be used as a measure of  
20 differential gene expression only in normalized samples. While empirical determination of the linear range of the amplification curve and normalization of cDNA preparations are tedious and time consuming processes, the resulting RT-PCR assays can be superior to those derived from the relative quantitative RT-PCR assay with an internal standard.

25 One reason for this advantage is that without the internal standard/competitor, all of the reagents can be converted into a single PCR product in the linear range of the amplification curve, thus increasing the sensitivity of the assay. Another reason is that with only one PCR product, display of the product on an electrophoretic gel or another display method becomes less complex, has less background and is easier to interpret.

## M. Methods for Activation of Gene Expression

In one embodiment of the present invention, there are provided methods for the increased gene expression or activation in a cell. This is particularly useful where there is  
5 an aberration in the gene product or gene expression is not sufficient for normal function. This will allow for the alleviation of symptoms of MODY3 type diabetes experienced as a result of mutation in HNF1 $\alpha$ , MODY4 type diabetes experienced as a result of mutation in HNF1 $\beta$  and MODY1 type diabetes experienced as a result of mutation in HNF4 $\alpha$ .

10 The general approach to increasing gene expression as mediated by HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  according to the present invention, will be to provide a cell with an HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  polypeptide, thereby permitting the transcription promotional activity of HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  to take effect. While it is conceivable that the protein may be delivered directly, a preferred embodiment involves providing a nucleic acid encoding an  
15 HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  polypeptide, *i.e.*, an HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  gene, to the cell. Following this provision, the HNF1 $\alpha$  HNF1 $\beta$  or HNF4 $\alpha$  polypeptide is synthesized by the host cell's transcriptional and translational machinery, as well as any that may be provided by the expression construct. Cis-acting regulatory elements necessary to support the expression of the HNF1 $\alpha$  HNF1 $\beta$  or HNF4 $\alpha$  gene will be provided, in the form of an  
20 expression construct. It also is possible that, expression of the virally-encoded HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  could be stimulated or enhanced, or the expressed polypeptide stabilized, thereby achieving the same or similar effect.

25 In order to effect expression of constructs encoding HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  genes, the expression construct must be delivered into a cell. One mechanism for delivery is via viral infection, where the expression construct is encapsidated in a viral particle which will deliver either a replicating or non-replicating nucleic acid. In certain embodiments an HSV vector is used, although virtually any vector would suffice.

Several non-viral methods for the transfer of expression constructs into cultured mammalian cells also are contemplated by the present invention. These include calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990) DEAE-dextran (Gopal, 1985), electroporation (Tur-Kaspa *et al.*, 1986; Potter *et al.*, 1984), direct microinjection (Harland and Weintraub, 1985), DNA-loaded liposomes (Nicolau and Sene, 1982; Fraley *et al.*, 1979) and lipofectamine-DNA complexes, cell sonication (Fechheimer *et al.*, 1987), gene bombardment using high velocity microprojectiles (Yang *et al.*, 1990), and receptor-mediated transfection (Wu and Wu, 1987; Wu and Wu, 1988). Some of these techniques may be successfully adapted for *in vivo* or *ex vivo* use, as discussed below.

In another embodiment of the invention, the expression construct may simply consist of naked recombinant DNA or plasmids. Transfer of the construct may be performed by any of the methods mentioned above which physically or chemically permeabilize the cell membrane. This is particularly applicable for transfer *in vitro*, but it may be applied to *in vivo* use as well. Another embodiment of the invention for transferring a naked DNA expression construct into cells may involve particle bombardment. This method depends on the ability to accelerate DNA coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them (Klein *et al.*, 1987). Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force (Yang *et al.*, 1990). The microprojectiles used have consisted of biologically inert substances such as tungsten or gold beads.

In a further embodiment of the invention, the expression construct may be entrapped in a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved

solutes between the lipid bilayers (Ghosh and Bachhawat, 1991). Also contemplated are lipofectamine-DNA complexes.

Liposome-mediated nucleic acid delivery and expression of foreign DNA in vitro  
5 has been very successful. Wong *et al.* (1980) demonstrated the feasibility of liposome-  
mediated delivery and expression of foreign DNA in cultured chick embryo, HeLa and  
hepatoma cells. In certain embodiments of the invention, the liposome may be complexed  
with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell  
membrane and promote cell entry of liposome-encapsulated DNA (Kaneda *et al.*, 1989). In  
10 other embodiments, the liposome may be complexed or employed in conjunction with  
nuclear non-histone chromosomal proteins (HMG-1) (Kato *et al.*, 1991). In yet further  
embodiments, the liposome may be complexed or employed in conjunction with both HVJ  
and HMG-1. In other embodiments, the delivery vehicle may comprise a ligand and a  
liposome. Where a bacterial promoter is employed in the DNA construct, it also will be  
15 desirable to include within the liposome an appropriate bacterial polymerase.

Other expression constructs which can be employed to deliver a nucleic acid  
encoding an HNF1 $\alpha$ , HNF1 $\beta$ , or HNF4 $\alpha$  transgene into cells are receptor-mediated delivery  
vehicles. These take advantage of the selective uptake of macromolecules by receptor-  
20 mediated endocytosis in almost all eukaryotic cells. Because of the cell type-specific  
distribution of various receptors, the delivery can be highly specific (Wu and Wu, 1993).

Receptor-mediated gene targeting vehicles generally consist of two components: a  
cell receptor-specific ligand and a DNA-binding agent. Several ligands have been used for  
25 receptor-mediated gene transfer. The most extensively characterized ligands are  
asialoorosomucoid (ASOR) (Wu and Wu, 1987) and transferrin (Wagner *et al.*, 1990).  
Recently, a synthetic neoglycoprotein, which recognizes the same receptor as ASOR, has  
been used as a gene delivery vehicle (Ferkol *et al.*, 1993; Perales *et al.*, 1994). Mannose can  
be used to target the mannose receptor on liver cells. Also, antibodies to CD5 (CLL), CD22  
30 (lymphoma), CD25 (T-cell leukemia) and MAA (melanoma) can similarly be used as

targeting moieties. In other embodiments, the delivery vehicle may comprise a ligand and a liposome.

Primary mammalian cell cultures may be prepared in various ways. In order for the  
5 cells to be kept viable while *in vitro* and in contact with the expression construct, it is necessary to ensure that the cells maintain contact with the correct ratio of oxygen and carbon dioxide and nutrients but are protected from microbial contamination. Cell culture techniques are well documented and are disclosed herein by reference (Freshner, 1992).

10 One embodiment of the foregoing involves the use of gene transfer to immortalize cells for the production of proteins. The gene for the protein of interest may be transferred as described above into appropriate host cells followed by culture of cells under the appropriate conditions. The gene for virtually any polypeptide may be employed in this manner. The generation of recombinant expression vectors, and the  
15 elements included therein, are discussed above. Alternatively, the protein to be produced may be an endogenous protein normally synthesized by the cell in question.

Examples of useful mammalian host cell lines are Vero and HeLa cells and cell  
20 lines of Chinese hamster ovary, W138, BHK, COS-7, 293, HepG2, NIH3T3, RIN and MDCK cells. In addition, a host cell strain may be chosen that modulates the expression of the inserted sequences, or modifies and process the gene product in the manner desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of  
25 protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. Appropriate cell lines or host systems can be chosen to insure the correct modification and processing of the foreign protein expressed.

A number of selection systems may be used including, but not limited to, HSV  
thymidine kinase, hypoxanthine-guanine phosphoribosyltransferase and adenine  
30 phosphoribosyltransferase genes, in *tk*-, *hprt*- or *aprt*- cells, respectively. Also, anti-

metabolite resistance can be used as the basis of selection for *dhfr*, that confers resistance to; *gpt*, that confers resistance to mycophenolic acid; *neo*, that confers resistance to the aminoglycoside G418; and *hygro*, that confers resistance to hygromycin.

5       Animal cells can be propagated *in vitro* in two modes: as non-anchorage dependent cells growing in suspension throughout the bulk of the culture or as anchorage-dependent cells requiring attachment to a solid substrate for their propagation (*i.e.*, a monolayer type of cell growth).

10       Non-anchorage dependent or suspension cultures from continuous established cell lines are the most widely used means of large scale production of cells and cell products. However, suspension cultured cells have limitations, such as tumorigenic potential and lower protein production than adherent cells.

15       Large scale suspension culture of mammalian cells in stirred tanks is a common method for production of recombinant proteins. Two suspension culture reactor designs are in wide use - the stirred reactor and the airlift reactor. The stirred design has successfully been used on an 8000 liter capacity for the production of interferon. Cells are grown in a stainless steel tank with a height-to-diameter ratio of 1:1 to 3:1. The 20 culture is usually mixed with one or more agitators, based on bladed disks or marine propeller patterns. Agitator systems offering less shear forces than blades have been described. Agitation may be driven either directly or indirectly by magnetically coupled drives. Indirect drives reduce the risk of microbial contamination through seals on stirrer shafts.

25

The airlift reactor, also initially described for microbial fermentation and later adapted for mammalian culture, relies on a gas stream to both mix and oxygenate the culture. The gas stream enters a riser section of the reactor and drives circulation. Gas disengages at the culture surface, causing denser liquid free of gas bubbles to travel 30 downward in the downcomer section of the reactor. The main advantage of this design is

the simplicity and lack of need for mechanical mixing. Typically, the height-to-diameter ratio is 10:1. The airlift reactor scales up relatively easily, has good mass transfer of gases and generates relatively low shear forces.

5      N.     **Methods for Blocking Mutant HNF1 $\alpha$ , HNF1 $\beta$  and HNF4 $\alpha$  Action**

In another embodiment of the present invention, there is contemplated the method of blocking the function of mutated HNF1 $\alpha$  in MODY3, HNF1 $\beta$  in MODY4, and HNF4 $\alpha$  in MODY1. In this way, it may be possible to curtail the effects of the mutation  
10     in diabetes. In addition, it may prove effective to use this sort of therapeutic intervention in combination with more traditional diabetes therapies, such as the administration of insulin.

The general form that this aspect of the invention will take is the provision, to a  
15     cell, of an agent that will inhibit mutated HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  function. Four such agents are contemplated. First, one may employ an antisense nucleic acid that will hybridize either to the mutated HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  gene or the mutated HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  gene transcript, thereby preventing transcription or translation, respectively. The considerations relevant to the design of antisense constructs have been  
20     presented above. Second, one may utilize a mutated HNF1 $\alpha$ -, HNF1 $\beta$ - or HNF4 $\alpha$ -binding protein or peptide, for example, a peptidomimetic or an antibody that binds immunologically to a mutated HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  respectively, the binding of either will block or reduce the activity of the mutated HNF1 $\alpha$ , HNF1 $\beta$  and HNF4 $\alpha$  respectively. The methods of making and selecting peptide binding partners and  
25     antibodies are well known to those of skill in the art. Third, one may provide to the cell an antagonist of mutated HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$ , for example, the transactivation target sequence, alone or coupled to another agent. And fourth, one may provide an agent that binds to the mutated HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  target without the same functional result as would arise with mutated HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  binding.

Provision of an HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  gene, a mutated HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  protein, or a mutated HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  antagonist, would be according to any appropriate pharmaceutical route. The formulation of such compositions and their delivery to tissues is discussed below. The method by which the nucleic acid, protein or chemical is transferred, along with the preferred delivery route, will be selected based on the particular site to be treated. Those of skill in the art are capable of determining the most appropriate methods based on the relevant clinical considerations.

Many of the gene transfer techniques that generally are applied *in vitro* can be adapted for *ex vivo* or *in vivo* use. For example, selected organs including the liver, skin, and muscle tissue of rats and mice have been bombarded *in vivo* (Yang *et al.*, 1990; Zelenin *et al.*, 1991). Naked DNA also has been used in clinical settings to effect gene therapy. These approaches may require surgical exposure of the target tissue or direct target tissue injection. Nicolau *et al.* (1987) accomplished successful liposome-mediated gene transfer in rats after intravenous injection.

Dubensky *et al.* (1984) successfully injected polyomavirus DNA in the form of CaPO<sub>4</sub> precipitates into liver and spleen of adult and newborn mice demonstrating active viral replication and acute infection. Benvenisty and Neshif (1986) also demonstrated that direct intraperitoneal injection of CaPO<sub>4</sub> precipitated plasmids results in expression of the transfected genes. Thus, it is envisioned that DNA encoding an antisense construct also may be transferred in a similar manner *in vivo*.

Where the embodiment involves the use of an antibody that recognizes a mutated HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  polypeptide, consideration must be given to the mechanism by which the antibody is introduced into the cell cytoplasm. This can be accomplished, for example, by providing an expression construct that encodes a single-chain antibody version of the antibody to be provided. Most of the discussion above relating to expression constructs for antisense versions of HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  genes will be relevant to

this aspect of the invention. Alternatively, it is possible to present a bifunctional antibody, where one antigen binding arm of the antibody recognizes an HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  polypeptide and the other antigen binding arm recognizes a receptor on the surface of the cell to be targeted. Examples of suitable receptors would be an HSV glycoprotein such as 5 gB, gC, gD, or gH. In addition, it may be possible to exploit the Fc-binding function associated with HSV gE, thereby obviating the need to sacrifice one arm of the antibody for purposes of cell targeting.

Advantageously, one may combine this approach with more conventional diabetes  
10 therapy options.

#### O. Pharmaceuticals and *In vivo* Methods for the Treatment of Disease

Aqueous pharmaceutical compositions of the present invention will have an effective amount of an HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  expression construct, an antisense HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  expression construct, an expression construct that encodes a therapeutic gene along with HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$ , a protein or compound that inhibits mutated HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  function respectively, such as an anti-mutant HNF1 $\alpha$  antibody, an anti-mutant HNF1 $\beta$  antibody or an anti-mutant HNF4 $\alpha$  antibody, or 20 a mutated HNF1 $\alpha$  polypeptide, mutated HNF1 $\beta$  polypeptide or a mutated HNF4 $\alpha$  polypeptide. Such compositions generally will be dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. An "effective amount," for the purposes of therapy, is defined at that amount that causes a clinically measurable difference in the condition of the subject. This amount will vary depending on the 25 substance, the condition of the patient, the type of treatment, the location of the lesion, etc.

The phrases "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other 30 untoward reaction when administered to an animal, or human, as appropriate. As used

herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is  
5 incompatible with the active ingredients, its use in the therapeutic compositions is contemplated. Supplementary active ingredients, such as other anti-diabetic agents, can also be incorporated into the compositions.

In addition to the compounds formulated for parenteral administration, such as  
10 those for intravenous or intramuscular injection, other pharmaceutically acceptable forms include, *e.g.*, tablets or other solids for oral administration; time release capsules; and any other form currently used, including cremes, lotions, mouthwashes, inhalants and the like.

The active compounds of the present invention will often be formulated for  
15 parenteral administration, *e.g.*, formulated for injection *via* the intravenous, intramuscular, subcutaneous, or even intraperitoneal routes. The preparation of an aqueous composition that contains mutated HNF1 $\alpha$ , HNF1 $\beta$  or HNF4 $\alpha$  inhibitory compounds alone or in combination with a conventional diabetes therapy agents as active ingredients will be known to those of skill in the art in light of the present disclosure.  
20 Typically, such compositions can be prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for using to prepare solutions or suspensions upon the addition of a liquid prior to injection can also be prepared; and the preparations can also be emulsified.

25 Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In many cases, the form must be sterile and must be

5 fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

The active compounds may be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts, include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium,

10 calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

The carrier also can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol,

20 sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle  
5 which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

10

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, with even drug release capsules and the like being employable.

15

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this  
20 connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 mL of isotonic NaCl solution and either added to 1000 mL of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some  
25 variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

P. Examples

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

**EXAMPLE 1**

**Altered Insulin Secretory Responses To Glucose In Diabetic And Nondiabetic Subjects With Mutations In The Diabetes Mellitus Susceptibility Gene MODY3 On Chromosome 12**

The present Example determines whether alterations in the dose-response relationships between plasma glucose concentration and insulin secretion rate (ISR) can be identified in subjects who have inherited an at-risk MODY3 allele but who have not yet developed overt diabetes.

**1. Methods**

*Subjects from MODY3 pedigrees*

Thirteen Caucasian subjects who were positive for MODY3 markers on chromosome 12q were studied. Two subjects were members of a French pedigree F549 (Vaxillaire *et al.*, 1995), three were from the P pedigree from Michigan (Menzel *et al.*, 1995), two from a New York pedigree the H pedigree depicted in FIG. 1, two were from a Liverpool pedigree, the BDA1 pedigree and four from a Nottingham pedigree, the BDA12 pedigree (FIG. 1). Each subject was typed with a series of DNA markers in the region of MODY3 to determine whether or not they had inherited the at-risk haplotype segregating with MODY in that family. The diabetes status of each subject except for

MD13, had been determined by oral glucose tolerance testing (OGTT) according to the World Health Organization (WHO) criteria (WHO Study Group on Diabetes Mellitus, 1985) and confirmed at the time of the studies by the measurement of glycosylated hemoglobin. Based on the results of the OGTT and glycosylated hemoglobin values 5 within or above the normal range for the inventors' laboratory (<7.4%) subjects were divided into diabetic and nondiabetic groups.

*Nondiabetic MODY3 subjects (n=6).*

The clinical profiles of these subjects are described in Table 4. All had normal 10 fasting glucose and glycosylated hemoglobin (<7.4%) levels at the time of this study. At the time of study 4 subjects had IGT (MD1, MD4, MD9, MD13) and 2 subjects had normal glucose tolerance (NGT) (MD3, MD5). Based on previous glucose tolerance testing MD1 had IGT, MD3 consistently demonstrated NGT on serial OGTTs, MD4 was diagnosed with IGT in 6/93 and has persistent IGT with a 2-h postprandial blood glucose 15 level of 147 mg/dl, MD5 was initially diagnosed with IGT and subsequently had 2 normal OGTTs, with 2-h blood glucose values of 130 mg/dl and 105 mg/dl, respectively, MD9 had IGT, with a 2-h post-challenge blood glucose level was 167 mg/dl with no other blood glucose level above 200 mg/dl and MD13 had IGT with elevated postprandial 20 blood glucose levels in the past up to 160 mg/dl. Age of diagnosis refers to the age at which abnormal glucose tolerance was diagnosed. None of these subjects were ever diagnosed with NIDDM.

*Diabetic MODY3 subjects (n=7).*

Clinical profiles are shown in Table 4. All subjects had been treated with oral 25 hypoglycemic agents except for MD8 who was taking insulin which was discontinued two days prior to the study and MD12 who was treated with diet alone. All subjects had discontinued treatment with oral hypoglycemic agents at least three weeks prior to being studied. As shown in Table 4, fasting plasma glucose and total glycosylated hemoglobin levels were higher in the diabetic group and fasting insulin levels were lower. The 30 diabetic group was also significantly older than the other two groups.

*Nondiabetic controls.*

The control subjects consisted of 5 males and one female (5 Caucasians and 1 African American) who did not have a personal or family history of NIDDM. They were 5 all within 20% of ideal body weight, had no medical illnesses and were not receiving any medications. Data from four of the control subjects have previously been published (Byrne *et al.*, 1994; Byrne *et al.*, 1995a). BMI was not significantly different between the control and diabetic or nondiabetic MODY3 groups.

10 Female volunteers had regular menstrual cycles and were studied only in the early follicular phase. The study was approved by the Institutional Review Board of the University of Chicago Medical Center and all subjects and/or parents provided written informed consent.

15 *Experimental protocol*

Studies began at 0800 h with subjects in the recumbent position after a 12-h overnight fast. An intravenous catheter was placed in each forearm, one for blood sampling and one for glucose administration. In all experiments, the arm containing the sampling catheter was maintained in a heating blanket or hot hand box to ensure 20 arterialization of the venous sample.

*Graded glucose infusion studies.*

These studies were designed to characterize the dose-response relationships between glucose and insulin secretion rate (ISR). In order to eliminate potentially 25 confounding effects of differences in the basal glucose concentration, each study began with the administration of a small bolus of insulin intravenously (0.007 U/kg) followed by a low dose continuous infusion of insulin to lower the fasting plasma glucose to similar levels in all groups (target plasma glucose = 5 mM). After a period of 20 min during which time the exogenously administered insulin was allowed to decay, samples 30 were drawn at 10 min intervals for 30 min to define baseline insulin, glucose and C-

peptide levels. An intravenous infusion of 20% dextrose was then started at a rate of 1 mg/kg/min, followed by infusions of 2 mg/kg/min, 3 mg/kg/min, 4 mg/kg/min, 6 mg/kg/min and 8 mg/kg/min. Each infusion rate was administered for a period of 40 min. Insulin, C-peptide and glucose concentrations were measured at 10, 20, 30 and 40 min  
5 into each infusion period.

*Effects of prolonged intravenous glucose administration on insulin secretory responses to graded glucose infusions.*

At the completion of the graded glucose infusion study described above, glucose  
10 was infused intravenously for a 42-h period at a rate of 4-6 mg/kg/min in order to determine if the insulin secretory responses to glucose could be primed by exposure to mild hyperglycemia. Subjects also consumed three carbohydrate enriched meals during the second day of this glucose infusion. At the conclusion of the 42-h infusion period, the infusion rate was reduced over a 60 min period and then stopped. Thirty minutes later,  
15 the graded glucose infusion study was repeated. Plasma glucose levels were obtained every four hours during the 42-h glucose infusion.

*Assays.*

Plasma glucose was measured by the glucose oxidase technique (YSI analyzer,  
20 Yellow Springs, OH). The coefficient of variation of this method is <2%. Serum insulin was assayed by a double antibody technique (Morgan and Lazarow, 1963). The average intra-assay coefficient of variation was 6%. Plasma C-peptide was measured as previously described (Faber *et al.*, 1978). The lower limit of sensitivity of the assay was 0.02 pmol/ml and the intra-assay coefficient of variation averaged 6%. All samples were  
25 measured in duplicate. Assays were performed at the University of Chicago.

*Data analysis*

*Estimation of ISRs.* ISRs were derived by deconvolution of plasma C-peptide concentrations assuming a two-compartmental model of C-peptide clearance kinetics  
30 (Van Cauter *et al.*, 1992; Eaton *et al.*, 1980; Polonsky *et al.*, 1986).

*Relationship between glucose and ISRs.*

The relationship between plasma glucose and ISR was explored in each individual by analyzing the data from the graded glucose infusion studies. Baseline glucose, insulin, C-peptide and ISRs were calculated as the mean of the values in the -30, -20, -10 and 0 min samples. During each glucose infusion period, average glucose and ISRs were calculated. Mean ISRs for each period were then plotted against the corresponding mean glucose level, thereby establishing a dose-response relationship between glucose and ISR. Mean ISRs were determined for 1 mM glucose concentration intervals by calculating the area under the curve for each interval using the trapezoidal rule. This area was divided by 1 mM to obtain the correct units (pmol/min).

*Statistical analyses*

All results are expressed as mean  $\pm$  SEM. Data analysis was performed using the Statistical Analysis System (SAS Version 6 Edition for Personal Computers, SAS Institute, Inc., Cary, NC). The significance of differences between the groups was determined using paired or unpaired *t*-tests or analysis of variance where appropriate. Tukey's studentized range test was used for post hoc comparisons. Pearson's correlation coefficient was used to evaluate correlations between pairs of parameters.

**2. Results**

*Glucose, insulin and ISR during graded intravenous glucose infusion*

Fasting plasma glucose levels were higher in the MODY3 diabetic group compared to the nondiabetic group or controls ( $7.5 \pm 0.7$  mM vs.  $4.5 \pm 0.2$  mM and  $4.7 \pm 0.2$ , respectively;  $P > 0.0008$ ). The corresponding fasting plasma insulin levels were lower in the diabetic MODY3 group compared to nondiabetics and controls (Table 4). Glucose, insulin and ISR responses to the glucose infusions are shown in FIG. 2A, FIG. 2B and FIG. 2C, respectively. Average glucose concentrations over the duration of the study were higher in the diabetic MODY3 subjects compared to the nondiabetic MODY3 and control subjects ( $8.5 \pm 0.4$  mM vs.  $6.3 \pm 0.3$  mM and  $64 \pm 0.2$ ;  $P < 0.0002$ ) (FIG. 2A). Average insulin levels were lower in the diabetic and nondiabetic MODY3 groups than in

the controls ( $57.4 \pm 8.2$  pmol/L and  $79.8 \pm 11.0$  vs.  $139.3 \pm 14.7$  pmol/L;  $P < 0.0006$ ) (FIG. 2B). Average ISR's were significantly lower in diabetic compared to the nondiabetic MODY3 subjects and the controls ( $116 \pm 18.8$  pmol/min vs.  $179.7 \pm 19.9$  pmol/min and  $1995 \pm 18.7$ ;  $P < 0.02$ ) (FIG.2C).

Non-Diabetic MODY 3

TABLE 4

ID	Kindred/Generation/ Glucose Tolerance		Sex	Age	BMI	Age of Diagnosis	Glucose mM	Insulin pmol/l	Fasting Glycohemoglobin
	Subject								
MD1	F549 IV-1	IGT	F	17.0	24.0	12	4.69	43.0	6.0
MD3	P IV-6	NGT	F	19.0	17.9		3.86	23.1	5.1
MD4	P IV-7	IGT	F	14.0	17.1	12	4.47	33.8	5.1
MD5	P IV-5	NGT	F	15.0	18.8	13	4.22	63.6	5.1
MD9	BDA1 V-12	IGT	M	14.0	20.0	12	4.77	69.9	6.4
MD13	BDA12 IV-2	IGT	F	14.0	23.7	12	5.17	60.9	6.4
Mean ± SEM				15.5±0.9	20.4±1.2		4.5±0.2	49.1±7.6	5.7±0.3
<b>Diabetic MODY3</b>									
MD2	F549 III-2	NIDDM	F	41	23.2	30	8.89	35.0	7.5
MD6	H IV-1	NIDDM	F	17	23.6	16	7.39	24.7	10.6
MD7	H IV-2	NIDDM	F	15	19.4	14	7.11	48.8	8.9
MD8	BDA1 V-11	NIDDM	F	17	20.9	12	4.22		7.6
M10	BDA 12 II-1	NIDDM	F	67	26.1	27	8.67	29.8	8.4
M11	BDA 12 IV-1	NIDDM	M	17	17.8	14	10.1	16.6	10.1
M12	BDA 12 III-2	NIDDM	F	46	21.4	14	6.19	43.0	7.6
Mean ± SEM				31.4±7.7	21.4±.9		7.51±0.7*	33±4.8*	8.7±0.5*
<b>Controls</b>									
Mean ± SEM				17.7±.2	21.1±0.7		4.7±0.2	69.9±8	<6.2
P value				p>0.08	p>0.8		P<0.0008	P<0.007	P<0.0004

Demographic data on the study subjects. Age of diagnosis refers to the age at which diabetes or IGT was diagnosed. MD3 is the only MODY3 subject who had demonstrated consistently normal glucose tolerance. p values refer to the results of analysis of variance comparing the three groups. The asterisks denote statistically significant differences between the diabetic subjects and the other two groups using Tukey's studentized range test for post-hoc comparisons.

TABLE 5

ID	Insulin Secreted between 5 and 9 mM glucose		
	Baseline	Post-glucose	Priming effect %
<b>Non-diabetic MODY3</b>			
MD1	188.1	221.6	17.9
MD3	164.5	255	55
MD4	136.6	208.3	52.5
MD5	297.5	342.5	15.1
MD9	249.1	292.1	34.5
MD13	248.1	234.2	-5.9
MEAN	214.3±24.8	259±20.6	35±8
<b>Diabetic MODY3</b>			
MD2	67.4	68.9	2.2
MD6	131.5	109.1	-17
MD7	144.6	85.2	-41
MD8	156.6	189.3	20.9
M10	63.7	34.9	-45
M11	38.2	28.4	-26
M12	102.6	115.1	12.2
MEAN	100.8±17.3*	90.0±20.8*	-13.4±9.8*
<b>Controls</b>			
C05	318.1	356.8	12.2
C07	209.5	272.1	29.2
C09	166.9	223.1	33.7
C12	235.6	381.6	62.0
C13	215.6	306.5	42.2
C18	120.1	180.5	50.3
MEAN	211±27	287±32	38±7
p value	p<0.004	P<0.002	p<0.009

5

The amount of insulin secreted as glucose was raised from 5 to 9 mM in study subjects before and after a priming intravenous infusion of glucose. Asterisks refer to statistically significant differences between the diabetic subjects and those in the other two groups using Tukey's studentized range test for post-hoc comparisons.

### *Changes in insulin sensitivity*

Insulin resistance estimated by the Homeostasis Model Assessment Method (HOMA) (Matthews *et al.*, 1985) failed to demonstrate significant differences between the groups (diabetic MODY3:  $1.9\pm0.2$ ; nondiabetic MODY3:  $1.7\pm0.3$ ; controls:  $2.4\pm0.2$ ;  $P=0.11$ ).

### *Dose-response relationship between glucose and ISR*

The ISR in the three groups was compared at the same plasma glucose level by plotting the mean ISR at each glucose infusion rate against the corresponding mean glucose level. The resulting glucose-ISR dose-response relationships are shown in FIG. 3. Over the 5-9 mM glucose concentration interval the diabetic MODY3 group secreted significantly less insulin than subjects in the nondiabetic MODY3 and control groups (101 $\pm$ 17 pmol/min vs. 214 $\pm$ 25 pmol/min and 211 $\pm$ 27 pmol/min, respectively;  $P<0.004$ ). The mean insulin secretion rate did not differ between these latter two groups.

The dose response curves (FIG. 3) indicate that the insulin secretion rates were similar in nondiabetic MODY subjects and controls at lower glucose concentrations. The amount of insulin secreted as the glucose concentration was increased from 5-7 mM was similar in these two groups (180 $\pm$ 19 vs. 160 $\pm$ 17 pmol/min;  $P=0.45$ ). Over the 7-8 mM glucose interval the nondiabetic MODY3 subjects secreted 243.5 $\pm$ 31.5 pmol/min compared to 284.7 $\pm$ 30.5 pmol/min in controls  $P=0.37$ . From 8-9 mM glucose they secreted 257.1 $\pm$ 35.0 pmol/min compared to 354.0 $\pm$ 43.4 pmol/min in controls  $P=0.12$  (FIG. 3). As the glucose concentration was increased from 7-8 mM to 8-9 mM the increase in insulin secretion rate in the nondiabetic MODY3 subjects was significantly less than in the controls (37.3 $\pm$ 13.5 vs. 75.7 $\pm$ 9.5 pmol/min;  $P<0.05$ ).

### *Effect of low-dose glucose infusion on relationships between glucose and ISR*

Mean glucose levels achieved during the 42-h constant glucose infusion were significantly higher in the diabetic compared to the nondiabetic MODY3 group and

controls ( $14.9 \pm 0.6$  mM vs.  $10.0 \pm 1.4$  mM vs.  $6.6 \pm 0.3$  mM;  $P < 0.0001$ ). The glucose infusion was discontinued after 42-h and low dose insulin was administered resulting in a fall in the plasma glucose concentration to similar levels in the two groups. The graded intravenous glucose infusion study was then repeated in each subject.

5

In order to quantify the priming effect of glucose on insulin secretion, the average ISR measured during each glucose infusion rate was plotted against the average plasma glucose concentration and compared with values obtained before glucose infusion. Over the glucose concentration range between 5 and 9 mM glucose, control subjects secreted  
10  $211 \pm 27$  pmol/min before and  $287 \pm 32$  pmol/min ( $P < 0.005$ ) insulin after glucose infusion (FIG. 4A). There was a shift in the glucose-ISR does-response curves upwards and to the left, with ISR increasing by  $38 \pm 7\%$ . The nondiabetic MODY3 group increased their ISR from  $214 \pm 25$  pmol/min to  $259 \pm 21$  pmol/min ( $P < 0.03$ ) (FIG. 4B). The diabetic MODY3 group had a small and non significant  $13 \pm 10\%$  decrease in ISR after glucose  
15 administration ( $101 \pm 17$  pmol/min to  $90 \pm 21$  pmol/min;  $P > 0.9$ ) (FIG. 4C). Individual values for ISR from 5-9 mM glucose before and after low-dose glucose infusion are given in Table 5.

20 *Relationship between glycosylated hemoglobin levels and parameters of the insulin secretory response to glucose*

There was a significant negative correlation between glycosylated hemoglobin and percent priming ( $r = -0.78$ ;  $P < 0.002$ ) and between glycosylated hemoglobin and ISR from 5-9 mM glucose ( $r = -0.61$ ;  $P < 0.03$ ). By contrast there was no significant decrease in ISR as glucose concentrations rose from 7-8 to 8-9 mM with increasing glycosylated  
25 hemoglobin levels ( $r = -0.07$ ;  $P = 0.82$ ).

3. Discussion

Basal glucose levels were higher and insulin levels were lower in MODY3 subjects with diabetes compared to nondiabetic subjects or normal healthy controls. In response to the graded glucose infusion, insulin secretion rates were significantly lower in the diabetic subjects over a broad range of glucose concentrations. Insulin secretion rates in the nondiabetic MODY3 subjects were not significantly different from the controls at plasma levels <8 mM. As glucose rose above this level, however, the increase in insulin secretion in these subjects was significantly reduced. Administration of glucose by intravenous infusion for 42-h resulted in a significant increase in the amount of insulin secreted over the 5-9 mM glucose concentration range in the controls and nondiabetic MODY3 subjects (by 38% and 35%, respectively) but no significant change was observed in the diabetic MODY3 subjects. In conclusion, in nondiabetic MODY3 subjects insulin secretion demonstrates a diminished ability to respond when blood glucose exceeds 8 mM. The priming effect of glucose on insulin secretion is preserved. Thus,  $\beta$ -cell dysfunction is present prior to the onset of overt hyperglycemia in this form of MODY. The defect in insulin secretion in the nondiabetic MODY3 subjects differ from than reported previously in nondiabetic MODY1 or mildly diabetic MODY2 subjects.

EXAMPLE 2

20 Mutations in HNF1 $\alpha$  Relating to MODY3 Type Diabetes

1. Materials and Methods

*Isolation of partial sequence of the human HNF1 $\alpha$  gene.*

The PAC clone, 254A7, containing the human HNF1 $\alpha$  gene was isolated from a library (Genome Systems, St. Louis, MO) by screening PAC DNA pools with PCR and the primers HNF1P1 (5'-TACACCACTCTGGCAGCCACACT-3' SEQ ID NO:10) and HNF1P2 (5'-CGGTGGGTACATTGGTGACAGAAC-3' SEQ ID NO:11). The sequences of the exons and flanking introns were determined after subcloning fragments of the 254A7 into pGEM-4Z (Promega Biotec, Madison, WI) or pBluescript SK+ (Stratagene, La Jolla, CA) and sequencing using primers based on the sequence of the human HNF1 $\alpha$

cDNA (Bach *et al.*, 1990; and Bach and Yaniv, 1993) and selected using the conserved exon-intron organization of the mouse and rat genes (Bach *et al.*, 1992) as a guide. Sequencing was carried using a AmpliTaq FS Dye Terminator Cycle Sequencing Kit (ABI, Foster City, CA) on an ABI Prism™ 377 DNA Sequencer (ABI). The sequences of the 5 exon 2/intron 2, exon 3/intron 3, intron 6/exon 7, and intron 8/exon 9/intron 9 junctions were determined by directly sequencing PCR products generated by amplification of PAC 254A7 or human genomic DNA. FIG. 11 shows the cDNA sequence of HNF1 $\alpha$ .

*Screening of HNF1 $\alpha$  gene for mutations.*

10 The ten exons and flanking introns of the HNF1 $\alpha$  gene of an affected subject from families in which of MODY cosegregated with markers spanning the MODY3 region of chromosome 12 subjects with the MODY3-form of NIDDM were amplified using PCR and specific primers (Table 6). PCR conditions were denaturation at 94°C for 5 min following by 35 cycles of denaturation at 94°C for 30 sec, annealing at 62°C for 30 sec  
15 (except for exon 9 - annealing temperature was 60°C) and extension at 72°C for 45 sec, and final extension at 72°C for 10 min. The PCR products were purified using a Centricon-100 membrane (Amicon, Beverly, MA) and sequenced from both ends using the primers shown in Table 6, a AmpliTaq FS Dye Terminator Cycle Sequencing Kit and ABI Prism™ 377 DNA Sequencer. The presence of the specific mutation in other family  
20 members was assessed by amplifying and directly sequencing the appropriate exon. At least 40 normal unrelated healthy non-diabetic non-Hispanic white subjects (80 chromosomes) were also similarly screened. DNA polymorphisms identified during the course of screening patients for mutations were characterized by PCR and direct sequencing, or digestion with an appropriate restriction endonuclease and gel  
25 electrophoresis.

**Table 6**  
 Sequences of primers used to amplify and directly sequence exons  
 and flanking introns of the human HNF1 $\alpha$  gene

Exon	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)
1	GGCAGGCCAACGCAAACCCACG (SEQ ID NO:12)	GAAGGGGGCTCGTTAGGAGC (SEQ ID NO:13)	483
2	CATGCACAGTCCCCACCCCTCA (SEQ ID NO:14)	CTTCAGCCCCACCTATGAG (SEQ ID NO:15)	384
3	GGGCAAGGTCAAGGGAAATGGA (SEQ ID NO:16)	CAGCCCAGACCAAACCAGCAC (SEQ ID NO:17)	306
4	CAGAACCCCTCCCCCTCATGCC (SEQ ID NO:18)	GGTGACTGTGTCAAATGGGAC (SEQ ID NO:19)	404
5	GCCTCCCTAGGGACTGCTCCA (SEQ ID NO:20)	GGCAGACAGGCAGATGGCCTA (SEQ ID NO:21)	347
6	TGGAGCAGTCCCTAGGGAGGC (SEQ ID NO:22)	GTTGCCCATGAGCCTCCAC (SEQ ID NO:23)	320

7	GGTCTGGCAGGGTGGAT (SEQ ID NO:24)	CTGCAATGCCTGCCAGGCACC (SEQ ID NO:25) CCCTGCATCCATTGACAGCC* (SEQ ID NO:26)	345
8	GAGGCCTGGACTAGGGCTGT (SEQ ID NO:27)	CTCTGTCACAGGCCAGGGAG (SEQ ID NO:28)	228
9	CCTGTGACAGAGCCCCTCAC (SEQ ID NO:29)	CGGACACAGCAACAGAAAGGGGTG (SEQ ID NO:31)	286
	CAGAGCCCCCTCACCCCCACAT*(SEQ ID NO:30)		
10	GTACCCCTAGGGACAGGCAGG (SEQ ID NO:32)	ACCCCCAAGCAGGCAGTACA (SEQ ID NO:33)	247

\* = primer used only for sequencing

## 2. Results

Table 7 identifies the DNA polymorphisms identified in the coding region of HNF1 $\alpha$  gene. Of course these are exemplary polymorphisms and those of skill in the art will easily be able to employ the methods and descriptions set forth in the present invention to identify other polymorphisms.

**Table 7.**  
DNA polymorphisms identified in coding region of human HNF1 $\alpha$  gene

Exon	Codon	Nucleotide change	Frequency
1	17	CTC(Leu) $\rightarrow$ CTG (Leu)	C, 0.57; G, 0.43
1	27	ATC(Ile) $\rightarrow$ CTC (Leu)	A, 0.63; C, 0.37
1	98	CCC(Ala) $\rightarrow$ GTC (Val)	C, 0.98; T, 0.02
4	279	GGG(Gly) $\rightarrow$ GGC (Gly)	G, 0.69; C, 0.31
7	459	CTG(Leu) $\rightarrow$ TTG (Leu)	C, 0.63; T, 0.37
7	487	AGC(Ser) $\rightarrow$ AAC (Asn)	G, 0.68; C, 0.32
8	515	ACG(Thr) $\rightarrow$ ACA(Thr)	G, 0.79; A, 0.21
Intron 1	nt-91	A $\rightarrow$ G	A, 0.88; G, 0.12
Intron 1	nt-42	G $\rightarrow$ A	G, 0.66; A, 0.34
Intron 2	nt-51	T $\rightarrow$ A	T, 0.85; A, 0.15
Intron 2	nt-23	C $\rightarrow$ T	C, 0.88; T, 0.12
Intron 5	nt-47	C $\rightarrow$ T	C, 0.99; T, 0.01
Intron 7	nt-7	G $\rightarrow$ A	G, 0.57; A, 0.43
Intron 9	nt-44	C $\rightarrow$ T	C, 0.96; T, 0.04
Intron 9	nt-24	T $\rightarrow$ C	T, 0.59; C, 0.41

10

Table 8 shows a summary of mutations identified in human HNF1 $\alpha$  in patients with MODY3. Sixteen exemplary mutations are identified in the HNF-1 $\alpha$  gene in MODY3 patients but were not present in unaffected individuals, these mutations include frameshifts

in exons 1, 4, 6, and 9, missense coding in exons 2, and 7 as well as abnormal splicing in introns 5 and 9. The results described herein demonstrate that mutations in this transcription factor can cause diabetes mellitus and focuses attention on the role of HNF-1 $\alpha$  in determining normal pancreatic  $\beta$ -cell function.

Table 8  
Summary Of Mutations In Human HNF1 $\alpha$  In Patients With MODY1

Location	Mutation/Location	Effect	Family
Exon 1	R55GfsdelGAGGG	Frameshift	F593
Exon 2	codon 122	Y→C	R213
	codon 131	R→Q	H,GL
	codon 142	S→F	F515
	codon 159	R→Q	F384
	codon 171	R→X	F Pierre
Exon 4	P291fsinsC	Frameshift	EA, SW, G17, G18, M13
	P291fsdelC	Frameshift	FS4
	G292fsdelG	Frameshift	F159
Intron 5	IVS5nt-2A→G	abnormal splice	P
Exon 6	P379fsdelCT	Frameshift	R,F632
	P379fsinsC	Frameshift	F549
	Q401fsdelC	Frameshift	G19
Exon 7	codon 447	P→L	A,Danish-1
Exon 9	T547E548fsdelTG	Frameshift	ber
Intron 9	IVS9nt+1G→A	abnormal splice	GK

### 3. Discussion

Linkage analysis localized *MODY3* to a 10 cM interval of chromosome 12 between the markers D12S86 and D12S342 (Vaxillaire *et al.*, 1995) and then to a 5 cM interval between the markers D12S86 and D12S807/D12S820 (Menzel, S. *et al.* 1995). A combined YAC, BAC and PAC contig spanning D12S86 and D12S807 (FIG. 9) was generated using information in public databases (Chumakov *et al.* 1995; Hudson *et al.* 1995) and screening appropriate libraries (YAC and BAC, Research Genetics, Huntsville, Alabama; and PAC, Genome Systems, St. Louis, Missouri) with STSs from the *MODY3* region. The physical map allowed localization of new polymorphisms as they were reported as well as to generate new markers to further localize recombination events in key individuals. Such studies refined the localization of *MODY3* to the 3 cM interval between D12S1666 and the polymorphic STS UC-39. Fluorescence in situ chromosomal hybridization using the BAC 162B15 mapped the contig to chromosome band 12q24.2.

15

This combination of genetic and physical mapping information was used to begin a systematic search for *MODY3*. Using a combination of approaches including testing genes known to be on the long arm of chromosome 12 to see if they mapped into the contig, exon-trapping (Church, *et al.* 1994), and cDNA selection (Kaplan *et al.*, 1992) using human pancreatic islet cDNA (clinical studies had shown that insulin secretion was abnormal in *MODY3* patients, and thus islets were a likely site of expression of *MODY3* mRNA and protein), the inventors identified 14 genes encoding known proteins ( $\gamma$ -subunit of AMP-activated protein kinase, citron, the GTP-binding protein H-ray, paxillin, acidic ribosomal phosphoprotein P0, pancreatic phospholipase A2, splicing factor SRp30, cytochrome C oxidase subunit VIa, short chain acyl CoA dehydrogenase, HNF-1 $\alpha$ , thyroid receptor interactor (TRIP14) protein, Ca $^{2+}$ /calmodulin-dependent protein kinase, P<sub>2</sub>X<sub>4</sub> purinoceptor and restin), 5 pseudogenes (metallopanstimulin-like, cell surface heparin binding protein-like, ribosomal protein L12-like, nucleoside diphosphate kinase-like and ADP ribosylation factor-like), 12 ESTs (yq81d09, yd50d03, IB383, hbc3028,

yu36h05, yn75d09, yz51b06, yd88g07, ym03h09, ym30e05, WI-6178/c-01h06, WI-6239/c-04b12) and 9 unknown genes (FIG. 9).

These genes were being systematically sequenced in affected and unaffected subjects using nested PCR and illegitimate transcription of lymphoblastoid RNA (Kaplan *et al.*, 1992), as well as PCR of individual exons of the gene. Comparison of the sequences of the pancreatic phospholipase A2,  $\gamma$ -subunit of AMP-activated protein kinase, H-ray, cytochrome C oxidase subunit VIA, acidic ribosomal phosphoprotein P0, paxillin, splicing factor SRp30, short chain acyl CoA dehydrogenase, and  $P_{2X4}$  purinoceptor genes from patients and controls revealed a number of polymorphisms but no MODY3-associated mutations.

The HNF-1 $\alpha$  gene was localized in the interval containing *MODY3* using PCR and HNF-1 $\alpha$  gene-specific primers (FIG. 9). HNF-1 $\alpha$  cDNAs were also isolated at high frequency by cDNA selection from human pancreatic islet cDNA using PAC 254A7, a result consistent with the report of Emens *et al.* (1992) showing that HNF-1 $\alpha$  was expressed in hamster insulinoma cells and functioned as a weak transactivator of the rat insulin I gene. The human HNF-1 $\alpha$  gene was isolated and partially sequenced to provide the exon-intron organization and the sequences of introns from which primers could be selected for PCR. The human gene consists of 10 exons with introns 1-8 located in the same positions as in the rat and mouse genes (Bach *et al.*, 1992). Intron 9 interrupts codon 590 (phase 1) and is not present in the rat and mouse genes but does occur in the chicken gene (Hörlein *et al.*, 1993) consistent with loss of this intron during the period when humans and rodents shared their last common ancestor. Amplification and direct sequencing of exon 4 of subject EA1 (Edinburgh pedigree, FIG. 5A) showed an insertion of a C in codon 289 (Pro) resulting in a frameshift and premature termination (designated P289fsinsC) (FIG. 10). This mutation was present in all affected members and no unaffected members of this family. It was also not found on screening 55 healthy non-diabetic white subjects (110 chromosomes). Hence it was concluded that the HNF-1 $\alpha$

gene is *MODY3* and led the inventors to sequence the HNF-1 $\alpha$  gene in other families in which NIDDM cosegregated with markers from the MODY3 region.

Fifteen additional mutations were found (Table 8), all of which co-segregated with NIDDM, and did not occur in any of at least 50 healthy non-diabetic white subjects. However, there were individuals in several pedigrees (GK pedigree, III-3; Ber pedigree, V-2; and P pedigree, IV-5 and IV-6) who had inherited the mutant chromosome (and at-risk chromosome 12 haplotype) but who were non-diabetic or showed only evidence of impaired glucose intolerance or diabetes during pregnancy. These individuals will likely develop NIDDM in the future. In addition, one subject with NIDDM did not have the mutant allele (Ber pedigree, II-2). He was diagnosed with NIDDM at 65 years of age at which time he was mildly obese with a body mass index of 27 kg/m<sup>2</sup> suggesting a diagnosis of late-onset NIDDM rather than MODY. Such heterogeneity within MODY families has been noted previously (Bell *et al.* 1991; Vionnet 1992) and is due to the high frequency of late-onset NIDDM which affects 10% or more of individuals over age 65 years (Kenny *et al.*, 1995). In addition to the mutations listed in Table 8, three amino acid polymorphisms (I/L27, A/V98 and S/N487), four silent polymorphisms (in codons for L17, G288, L459 and T515) and seven polymorphisms in introns were found in the HNF-1 $\alpha$  gene (Tables 7 and 8).

20

Sixteen different mutations in the HNF-1 $\alpha$  gene were identified in patients with the MODY3-form of diabetes. The splicing and frameshift mutations would be predicted to result in the expression of a truncated protein having at least amino acids 1-290 of the native protein. The missense mutations, R131Q and P447L, are of residues that are conserved in human, rat, mouse, hamster, chicken, *Xenopus* and salmon HNF-1 $\alpha$  and the structurally-related transcription factor human HNF-1 $\beta$  suggesting that these residues are functionally important.

30 HNF-1 $\alpha$  is one of a group of transcription factors expressed in liver that act together to confer tissue-specific expression of genes in this tissue (Tronche *et al.*, 1992;

Bach *et al.*, 1990). It is also found in kidney, intestine, stomach and pancreas, including islets of Langerhans, and at low levels in spleen and testis suggesting that it plays a role in transcriptional regulation in these tissues as well. HNF-1 $\alpha$  is composed of three functional domains: an NH<sub>2</sub>-terminal dimerization domain (amino acids 1-32), a DNA binding domain with POU-like and homeodomain-like motifs (amino acids 150-280) and a COOH-terminal transactivation domain (amino acids 281-631). The functional form of HNF-1 $\alpha$  is a dimer and HNF-1 $\alpha$  may form homodimers or heterodimers with the structurally-related protein HNF-1 $\beta$  (Mendel *et al.*, 1991)

Pontoglio *et al.* (1996) have generated mice that lack HNF-1 $\alpha$ . Homozygous HNF-1 $\alpha$ -deficient animals failed to thrive and usually died around the time of weaning. They also suffered from phenylketonuria and renal tubular dysfunction. However, the homozygous HNF-1 $\alpha$ -deficient mice did not appear to be diabetic as they had normal blood glucose levels and a normal response to an intravenous bolus injection of glucose. The massive glucosuria in these animals though may have masked the presence of diabetes mellitus. The insulin secretory responses of heterozygous HNF-1 $\alpha$ -deficient mice, animals that may be most similar to human subjects with HNF-1 $\alpha$  mutations and MODY, were not reported. In view of the present findings that mutations in the HNF-1 $\alpha$  gene causes early-onset NIDDM, more detailed evaluation of  $\beta$ -cell and liver function in HNF-1 $\alpha$ -deficient mice is indicated.

The mechanism by which mutations in the HNF-1 $\alpha$  gene when present on a single allele can cause diabetes is unclear however, it is possible that a partial deficiency of HNF-1 $\alpha$  could lead to  $\beta$ -cell dysfunction and diabetes. Alternatively, mutations in HNF-1 $\alpha$  may cause diabetes by a dominant-negative mechanism (Herskowitz, 1987) by interfering with the function of wild-type HNF-1 $\alpha$  and other proteins which act in concert with HNF-1 $\alpha$  to regulate transcription in the  $\beta$ -cell and/or liver. All of the HNF-1 $\alpha$  gene mutations identified to date would result in the synthesis of a mutant protein impaired in DNA binding or transactivation but not dimerization. These mutant proteins could form

non-productive dimers with the product of the normal HNF-1 $\alpha$  allele or other proteins such as HNF-1 $\beta$  and thereby impair the normal function of HNF-1 $\alpha$ .

The inventors have previously shown that diabetes mellitus in the Zucker diabetic fatty rat, a rodent model of obesity and NIDDM, is associated with decreased expression of a large number of  $\beta$ -cell genes including genes such as insulin whose expression is restricted to the  $\beta$ -cell as well as others with a much broader tissue distribution (Tokuyama, *et al.* 1995). Thus, it is believed that NIDDM is likely to be a disorder of transcription with genetic or acquired defects affecting key proteins that regulate transcription leading to  $\beta$ -cell dysfunction and diabetes.

### EXAMPLE 3 Mutations in HNF4 $\alpha$ Relating to MODY1 Type Diabetes

The PAC clone, 114E13, 130B8, 207N8, containing the human HNF4 $\alpha$  gene was isolated from a library (Genome Systems, St. Louis, MO) by screening PAC DNA pools with PCR and the primers HNF4P1 (5'-CACCTGGTGATCACGTGGTC-3' SEQ ID NO:81) and HNF4P2 (5'-GTAAGGCTCAAGTCATCTCC-3' SEQ ID NO:82). The sequences of the exons and flanking introns were determined by directly sequencing using primers based on the sequence of the human HNF4 $\alpha$  cDNA (Chartier *et al.*, 1994; Drewes *et al.*, 1996) and selected using the conserved exon-intron organization of the mouse (Taraviras *et al.*, 1994) as a guide. Sequencing was carried using a AmpliTaq FS Dye Terminator Cycle Sequencing Kit (ABI, Foster City, CA) on an ABI Prism TM 377 DNA Sequencer (ABI).

*Screening of HNF4 $\alpha$  gene for mutations.*

The eleven exons and flanking introns of the HNF4 $\alpha$  gene of an affected subject from families in which of MODY cosegregated with markers spanning the MODY1 region of chromosome 20 subjects with the MODY1-form of NIDDM were amplified using PCR and specific primers (Table 9). PCR conditions were denaturation at 94°C for

5 min following by 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec, and final extension at 72°C for 10 min. The PCR products were purified using a Centricon-100 membrane (Amicon, Beverly, MA) and sequenced from both ends using the primers shown in Table 9, a AmpliTaq FS Dye  
5 Terminator Cycle Sequencing Kit and ABI Prism™ 377 DNA Sequencer. The presence  
of the specific mutation in other family members was assessed by digestion with Bta3  
restriction endonuclease that resulted from mutation and gel electrophoresis. At least 100  
normal unrelated healthy non-diabetic non-Hispanic white subjects (200 normal  
chromosomes) were also similarly screened. DNA polymorphisms identified during the  
10 course of screening patients for mutations were characterized by PCR and direct  
sequencing, or digestion with an appropriate restriction endonuclease and gel  
electrophoresis.

Table 9. DNA Sequences of PCR Primers for MODY1

Exon	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)
1	GGGCACTGGGAGGGCAGT (SEQ ID NO:56)	GCCTGTAGGACCAACCTACC (SEQ ID NO:57)	340
1b	TCTGGTTGCGACGACTGCAC (SEQ ID NO:58)	CTGGAGCTGGAGGCTCATAC (SEQ ID NO:59)	356
2	AAGGCTCCCTTAGATGCCCTG (SEQ ID NO:60)	CCACTCAGGGAGAAAGACAGACCT (SEQ ID NO:61)	321
3	CCTAGTTCTGTCCTAAGAGG (SEQ ID NO:62)	GTCATAAAAGTGTGGCTACAG (SEQ ID NO:63)	253
4	CCACCCCCCTACTCCATCCCCGT (SEQ ID NO:64)	CCCTCCCCGTCAAGCTGCCCA (SEQ ID NO:65)	272
5	GTGCAGGGACAGAGAACATGC (SEQ ID NO:66)	AATCAAGCCAGTCCACGGCTAT (SEQ ID NO:67)	322
6	GCCCAGCGTCACTGAGTTGGCTA (SEQ ID NO: 68)	TTGCCTGGGTGAGTGCCATG (SEQ ID NO:69)	234
7	GCACCAGCTATCTTGCCAAC (SEQ ID NO:70)	AGGAGAAAGTCTGGCAGAGCG (SEQ ID NO:71)	315
8	CTCCTTGTGTGACACAAGTC (SEQ ID NO:72)	CTCACTGTGTGAGGGCTGTG (SEQ ID NO:73)	407
9	TGGTTGATTGGCCACGGCTG (SEQ ID NO:74)	ATCCTGGTTCTACCTTCTAG (SEQ ID NO:75)	341
10	CATTTACTCCCCACAAAGGCT (SEQ ID NO:76)	GACCACGTGATCACCAAGGTG (SEQ ID NO:77)	277

Table 10 identifies the DNA polymorphisms and mutations identified in the coding region of the HNF4 $\alpha$  gene. Of course, these are exemplary polymorphisms and those of skill in the art will easily be able to employ the methods and descriptions set forth in the present invention to identify other polymorphisms. FIG. 7 shows an alignment of the HNF4 $\alpha$  protein sequence from humans with sequences from human mouse, X. Laves and Drosophila. The putative DNA binding sites are underlined and the putative ligand binding sites are in bold. The DNA sequences for exon 1, exon 1b, exon 2, exon 3, exon 4, exon 5 exon 6 exon 7 exon 8 exon 9 and exon 10 of HNF4 $\alpha$  are shown in FIG. 8A, FIG. 8B, FIG. 8C, FIG. 8D FIG. 8E, FIG. 8F, FIG. 8G, FIG. 8I, FIG. 8H, FIG. 8I and SEQ ID NO:34, SEQ ID NO:36, SEQ ID NO:38, SEQ ID NO:40, SEQ ID NO:42, SEQ ID NO:44, SEQ ID NO:46, SEQ ID NO:48, SEQ ID NO:50, SEQ ID NO:52, and SEQ ID NO:54, respectively. It is contemplated that mutations in any of these exons, or the related intron regions therebetween, of HNF4 $\alpha$  will result in MODY1 type diabetes.

15

**Table 10.**  
Polymorphisms and Mutations in the Human HNF4 $\alpha$  Gene

Location	Nucleotide change		Frequency
Exon	Codon		
4	130	ACT (Thr)-ATT(Ile)	C:T=105:5 C-0.95, T-0.05
7	273	GAT(Asp)-GAC(Asp)	T:C=169:1 T-0.004, C-0.006
7	268	GAG(Gln)-TAG(stop)	0/216 control chromosomes

20

The R-W pedigree, which includes more than 360 members spanning 6 generations and 74 members with diabetes including those with MODY, has been studied prospectively since 1958 (Fajans, 1989). The members of this family are descendants of a man who was born in East Prussia in 1809 and emigrated to Detroit, Michigan in 1861 25 with his four sons, three of whom were diabetic, and five daughters, one of whom was diabetic (Fajans, 1989; Fajans *et al.*, 1994). Linkage studies have shown that the gene responsible for MODY in this family, *MODY1*, is tightly linked to markers in chromosome band 20q12-q13.1 with a multipoint lod score >14 in those branches of the

family in which MODY is segregating (Bell, *et al.* 1991; Bowden, *et al.*, 1992; Irwin, *et al.*, 1994). The analysis of key recombinants in the R-W pedigree localized *MODY1* to a 13-cM interval (~7 Mb) between D20S169 and D20S176, an interval which also includes the gene encoding HNF-4 (Stoffel, M. *et al.*, 1996). The demonstration in the previous 5 examples that mutations in the HNF-1 $\alpha$  gene are the cause of the MODY3-form of NIDDM prompted the inventors to screen the HNF-4 $\alpha$  gene for mutations in the R-W pedigree.

The human HNF-4 $\alpha$  gene consists of 11 exons with the introns being located in 10 the same positions as in the mouse gene (Tavaviras, *et al.*, 1994). Alternative splicing generates a family of HNF-4 $\alpha$  mRNAs, HNF-4 1, 2 and 4, the latter two of which contain inserts of 30 and 90 nucleotides, respectively (Tavaviras *et al.*, 1994; Laine *et al.*, 1994; Drewes, 1996). Of these, HNF-4 2 mRNA appears to be the most abundant transcript in many tissues. In contrast to a previous report (Drewes *et al.*, 1996), the inventors studies 15 show that HNF-4 $\alpha$  mRNA encodes a truncated and presumably nonfunctional form of HNF-4 $\alpha$ . The sequence of exon 1B, the exon encoding the insertion in HNF-4 $\alpha$  mRNA revealed an additional T between nucleotides 219 and 220 in both alleles of five unrelated individuals (10 chromosomes) not present in the cDNA sequence (Drewes *et al.*, 1996) which causes a frameshift and the generation of a protein of 98 amino acids whose 20 function, if any, is unknown. The 11 exons of the HNF-4 $\alpha$  gene of two affected, V-20 and 22, and one unaffected, VI-9, subject from the R-W pedigree were amplified and the PCR products sequenced directly. The sequences were identical to one another and to the cDNA (Drewes *et al.*, 1996; Laine *et al.*, 1994)) except for a C→T substitutions in exon 25 4, codon 130 and exon 7, codon 268. The C→T substitution in codon 130 results in a Thr (ACT)→Ile (ATT) substitution and is a polymorphism (T/I130) with a frequency of the Ile allele in a group of 55 unrelated nondiabetic non-Hispanic white subjects of 5%. The C→T substitution in codon 268 results in a nonsense mutation CAG (Gln)→TAG (AM) (Q268X). The nonsense mutation was confirmed by cloning and sequencing PCR products derived from both alleles. The Q268X mutation created a site for the enzyme

*Bfa* I with digestion of the normal allele generating fragments of 281 and 34 bp, and the mutant allele, 152, 129 and 34 bp and facilitating testing for this mutation in other members of the R-W pedigree. In the R-W pedigree, Ile130 and the amber mutation at codon 268 were present in the same allele.

5

The Q268X mutation cosegregated with the at-risk haplotype and NIDDM in the R-W pedigree and was not observed on screening 108 healthy nondiabetic non-Hispanic white subjects (216 normal chromosomes). Seven subjects in the R-W pedigree who have inherited the mutant allele (V-18, 37 and 48; and VI-6, 11, 15 and 20) have normal glucose tolerance. The ages of five of these subjects (V-48, and VI-6, 11, 15 and 20) are less than 25 years and thus, they are still within the age range when diabetes usually develops in at-risk individuals in this family. Of the others, subject V-18 is 44 years of age and has shown normal glucose on all oral glucose tolerance tests, and subject V-37 who is 36 years of age had one glucose tolerance test characteristic of impaired glucose tolerance and one of diabetes at ages 16-17 years but for the past 19 years each glucose tolerance test has been normal even though she has a low insulin response to orally administered glucose. She is very lean and active, and has increased sensitivity to insulin during the frequently sampled intravenous glucose tolerance test. During a prolonged low dose glucose infusion, she became markedly hyperglycemic (Herman, *et al.* 1994; Byrne, *et al.* 1995). Two subjects (V-1 and 4) who have the mutation were considered nondiabetic based on medical history and their affection status needs to be evaluated by oral glucose tolerance testing. The results indicate that the nonsense mutation in the HNF-4 gene in the R-W pedigree is highly but not completely penetrant although the age of diabetes onset is variable.

25

In addition to subjects who inherited the Q268X mutation but are presently nondiabetic, there are subjects in the R-W pedigree who have NIDDM but did not inherit the Q268X mutation or at-risk haplotype. Subject IV-9 was diagnosed with NIDDM at 48 years of age and was hyperinsulinemic, a diagnosis consistent with late-onset NIDDM rather than MODY. The inventors also tested her six children, one of whom had NIDDM

and another impaired glucose tolerance, and all had two normal alleles. Similarly, 10 children of subject III-7, five of whom had NIDDM were also tested, and none had inherited the Q268X mutation, suggesting that the NIDDM in this branch of the R-W family is of a different etiology. Finally, the five nondiabetic children of III-11 were also 5 tested and all were normal. The presence of both MODY and late-onset NIDDM in the R-W family has been noted previously (Bell, *et al.* 1991; Bowden, *et al.*, 1992). The MODY phenotype results from a mutation in the HNF-4 gene. The cause(s) of the late-onset NIDDM is unknown.

10 HNF-4 is a member of the steroid/thyroid hormone receptor superfamily and is expressed at highest levels in liver, kidney and intestine (Xanthopoulos *et al.*, 1991; Sladek *et al.*, 1990). It is also expressed in pancreatic islets and insulinoma cells (Miquerol, *et al* 1994). In liver, HNF-4 $\alpha$  is a key regulator of hepatic gene expression and is a major activator of HNF-1 $\alpha$  which in turn activates expression of a large number 15 of liver-specific genes including those involved in glucose, cholesterol and fatty acid metabolism (Sladek *et al.*, 1990; Kuo *et al.*, 1992). Its expression in kidney, intestine and pancreatic islets implies that it plays a central role in tissue-specific regulation of gene expression in these tissues as well, although its specific function in nonhepatic tissues has not been addressed. Homozygous loss of functional HNF-4 $\alpha$  protein causes embryonic 20 lethality characterized by defects in gastrulation underscoring the key role played by this transcription factor in development and differentiation (Chen *et al.*, 1994). The phenotype of the heterozygous animals was not described and further studies are necessary to determine if they represent a mouse model of MODY.

25 HNF-4 $\alpha$  defines a subclass of nuclear receptors which reside primarily in the nucleus and bind to their recognition site and regulate transcription as homodimers (Sladek *et al.*, 1994; Kuo *et al.*, 1992). The key role played by HNF-4 $\alpha$  in the regulation of hepatic gene expression is well established (Sladek *et al.*, 1994; Kuo *et al.*, 1992). However, its role as well as that of HNF-1 $\alpha$ , the MODY3 product and a downstream 30 target of HNF-4 $\alpha$  action, in regulating gene expression in the insulin-secreting pancreatic

$\beta$ -cell is largely unknown, although Emens *et al.*(1992) have shown that HNF-1 $\alpha$  is a weak transactivator of the insulin gene. Thus, the mechanism by which mutations in HNF-4 $\alpha$  result in an autosomal dominant form of NIDDM characterized by pancreatic - cell dysfunction is unclear. The nonsense mutation in HNF-4 $\alpha$  found in the R-W family  
5 is predicted to result in the synthesis of a protein of 267 amino acids with an intact DNA binding domain. However, it is missing the regions involved in dimerization and transcriptional activation in other members of the steroid/thyroid hormone superfamily  
Zhang, *et al.*, 1994; Bourguet, *et al.*, 1995; Renaud, *et al.* 1995; Wagner, R.L. *et al.* 1995)  
and as a consequence is predicted to be unable to dimerize, bind to its recognition site and  
10 activate transcription. Thus, the dominant inheritance is due to a reduction in the amount  
of HNF-4 $\alpha$  *per se* rather than a dominant negative mechanism. The decreased levels of  
functional HNF-4 $\alpha$  appear to have a critical effect on  $\beta$ -cell function perhaps as a  
consequence of decreased HNF-1 $\alpha$  gene expression, mutations in this gene also leading  
15 to MODY as described in the examples above. Prediabetic subjects with mutations in  
either the HNF-4 $\alpha$  or HNF-1 $\alpha$  genes exhibit similar abnormalities in glucose-stimulated  
insulin secretion with normal insulin secretion rates at lower glucose concentrations but  
lower than normal rates as the glucose concentration increases (Byrne *et al.*, 1995), a  
result consistent with HNF-4 $\alpha$  and HNF-1 $\alpha$  affecting a common pathway in the  
pancreatic  $\beta$ -cell. The absence of overt hepatic, renal or gastrointestinal dysfunction in  
20 affected members of the R-W pedigree suggests that the levels of HNF-4 $\alpha$  in these  
tissues, although possibly lower than normal, are sufficient to ensure normal function or  
that alternative pathways are sufficient for expression of key genes. However, detailed  
studies of hepatic glucose production and metabolism have not performed in subjects  
from the R-W pedigree and it is possible that subtle alterations in these processes may be  
25 present.

The demonstration that MODY can result from mutations in the HNF-1 $\alpha$  and HNF-4 $\alpha$  genes suggests that this form of NIDDM is primarily a disorder of abnormal gene expression. In this regard, genes encoding other proteins in the HNF-1 $\alpha$ /HNF-4 $\alpha$

regulatory cascade such as other members of the HNF-1 (Mendel *et al.*, 1994) and HNF-4 families (Drewes *et al.*, 1996) as well as HNF-3 (Lai *et al.*, 1993), HNF-6 (Lemaigre, *et al.* 1996.), and perhaps dimerization cofactor of HNF-1 (Mendel *et al.*, 1991) should be considered as candidates for other forms of MODY and/or late-onset NIDDM. The role  
5 of HNF-4 $\alpha$  in the development of the more common late-onset NIDDM is unknown. There is no evidence for linkage of markers flanking the HNF-4 $\alpha$  gene with late-onset NIDDM in Mexican Americans or Japanese implying that mutations in the HNF-4 $\alpha$  gene are unlikely to a significant genetic factor contributing to the development of late-onset NIDDM. However, acquired defects in HNF-4 $\alpha$  expression may contribute, at least in  
10 part, to the  $\beta$ -cell dysfunction which characterizes late-onset NIDDM (Polonsky *et al.*, 1996) especially if it plays a central role in regulating gene expression in the pancreatic  $\beta$ -cell as suggested by its association with MODY. Furthermore, the similarity between HNF-4 $\alpha$  and ligand dependent transcription factors raises the possibility that HNF-4 $\alpha$  and the genes it regulates respond to an unidentified ligand. The identification of such a  
15 ligand by the methods of the present invention will lead to new approaches for treating diabetes.

#### EXAMPLE 4

20      **Organization and Partial Sequence of the HNF 4 $\alpha$ /MODY1 Gene and  
Identification of Missense Mutation, R127W, in a Japanese Family with MODY**

HNF-4 $\alpha$  is a member of the nuclear receptor superfamily, a class of ligand-activated transcription factors. A nonsense mutation in the gene encoding this transcription factor has been recently found in a white family with one form of maturity-onset diabetes of the young, MODY1. In the present example, the inventors report the exon-intron organization and partial sequence of the human HNF-4 $\alpha$  gene. In addition,  
25 the inventors have screened the twelve exons, flanking introns and minimal promoter region for mutations in a group of 57 unrelated Japanese subjects with early-onset NIDDM/MODY of unknown cause. Eight nucleotide substitutions were noted, of which

one resulted in the mutation of a conserved arginine residue, Arg127 (CGG)→Trp (TGG) (designated R127W), located in the T-box, a region of the protein that may play a role in HNF-4 $\alpha$  dimerization and DNA binding. This mutation was not found in 214 unrelated nondiabetic subjects (53 Japanese, 53 Chinese, 51 white and 57 African-American). The  
5 R127W mutation was only present in three of five diabetic members in this family indicating that it is not the only cause of diabetes in this family. The remaining seven nucleotide substitutions were located in the proximal promoter region and introns. They are not predicted to affect the transcription of the gene or mRNA processing and represent polymorphisms and rare variants. The results suggest that mutations in the HNF-4 $\alpha$  gene  
10 may cause early-onset NIDDM/MODY in Japanese but they are less common than mutations in the HNF-1 $\alpha$  /MODY3 gene. The information on the sequence of the HNF-4 $\alpha$  gene and its promoter region will facilitate the search for mutations in other populations and studies of the role of this gene in determining normal pancreatic  $\beta$ -cell function.

15 **1. Methods**

*Isolation and partial sequence of the human HNF-4 $\alpha$  gene*

Three P1-derived artificial chromosome (PAC) clones, 114E13, 130B8 and 207N8, containing the human HNF-4 $\alpha$  gene were isolated by screening PAC DNA pools (Genome System, St. Louis, MO) by PCR™ with HNF-4 $\alpha$  specific primers (Yamagata *et* 20 *al.*, 1996a). The partial sequence of the HNF-4 $\alpha$  gene was determined using DNA from PAC's 114E13 and 207N8 and sequence-specific primers with an AmpliTaq FS Dye Terminator Cycle Sequencing Kit and ABI Prism™ 377 DNA sequencer (ABI, Foster City, CA). The promoter sequence was examined for transcription factor binding sites using MatInspector (Quandt *et al.*, 1995) and TFSEARCH (Version 1.3 25 http://www.genome.ad.jp/kit/tfsearch.html). The sequences of alternatively-spliced mRNAs were confirmed by sequencing PCR™ products generated by amplification of human liver cDNA using specific primers.

*Screening of the HNF-4 $\alpha$  gene for mutations*

The 12 exons, flanking introns and minimal promoter region were screened for mutations by amplifying and directly sequencing both strands of the PCR™ product using specific primers (the sequences of the primers are available at www.diabetes.org/diabetes). The sequence of the missense mutation (R127W) was confirmed by cloning the PCR™ product into pGEM-T (Promega, Madison, WI) and sequencing clones representing both alleles. The R127W mutation leads to loss of a *Msp* I site and subjects were tested for the presence of this mutation by digestion of the PCR™ product of exon 4 with *Msp* I, separation of the fragments by electrophoresis on a 3% NuSieve® 3:1 agarose gel (FMC BioProducts, Rockland, ME) and visualization by ethidium bromide staining. The sequences of the DNA polymorphisms are based on sequencing both strands of the PCR™ product and were not confirmed directly by cloning and sequencing the PCR™ product.

15           *Subjects*

The study population consisted of 57 unrelated Japanese subjects attending the Diabetes Clinic, Tokyo Women's Medical College who were diagnosed with NIDDM before 25 years of age and/or who were members of families in which NIDDM was present in three or more generations: age at diagnosis,  $20.1 \pm 7.5$  years (mean $\pm$ SE); male/female, 31/26; and treatment, insulin - 36, oral hypoglycemic agents - 10, and diet - 11. Thirty-two of the subjects met strict criteria for a diagnosis of MODY (*i.e.*, NIDDM in at least three generations with autosomal dominant transmission and diagnosis before 25 years of age in at least one affected subject). NIDDM was diagnosed using the criteria of the World Health Organization (Bennett *et al.*, 1994). At the time of recruitment, informed consent was obtained from each subject and a blood sample was taken for DNA isolation. Fifty-three unrelated nondiabetic Japanese subjects were tested for each nucleotide substitution and mutation to determine if the sequence change was a polymorphism or disease-associated mutation. In addition, 53 Chinese (15), 51 white (16), and 57 African-American unrelated nondiabetic subjects (16) were tested for the R127W mutation.

## 2. Results

Organization and partial sequence of human HNF-4 $\alpha$  gene. The human HNF-4 $\alpha$  gene (gene symbol, *TCF14*) consists of 12 exons spanning approximately 30 kb, of which about 10 kb were sequenced including 1 kb of the promoter region (the gene sequence is available at [www.diabetes.org/diabetes](http://www.diabetes.org/diabetes)). Human HNF-4 $\alpha$  mRNA is alternatively spliced (Hata *et al.*, 1992; Chartier *et al.*, 1994; Drewes *et al.*, 1996; Kritis *et al.*, 1996) which may generate as many as six different forms of HNF-4 $\alpha$  (FIG. 12). HNF-4 $\alpha$ 2 is the predominant form present in many adult tissues including liver, kidney and intestine. The inventors have used RT-PCR<sup>TM</sup> to determine which HNF-4 $\alpha$  transcripts are expressed in human pancreatic islets. This analysis showed that islets express mRNAs for HNF-4 $\alpha$ 1, 2 and 3. The inventors could not detect islet transcripts that included exons 1C and 1B although transcripts containing these two exons could be detected in human liver by RT-PCR<sup>TM</sup>.

The sequence of 1 kb of the promoter region of the human HNF-4 $\alpha$  gene was determined (FIG. 13). The comparison of the sequences of the human and mouse genes showed regions of sequence conservation that included the predicted start of transcription and the binding sites for several transcription factors including HNF-6, AP-1, HNF-3, HNF-1 $\alpha$  and NF-1. The transcription start site for the human gene has not been determined directly but has been inferred from studies of the mouse gene which showed multiple start sites spread over a 10 bp interval (Zhong *et al.*, 1994; Tavaviras *et al.*, 1994) of which one was defined as nucleotide +1 (Zhong *et al.*, 1994). The sequence homology in the promoter of the human and mouse genes suggests that transcription of the HNF-4 $\alpha$  gene may be regulated in a similar manner. In this regard, Zhong *et al.* (Zhong *et al.*, 1994) have shown that the major promoter activity in a hepatoma cell line was associated with a 126 bp fragment of the mouse promoter (nucleotides 289-414 in FIG. 13). There is 83% identity between the human and mouse sequences in this minimal promoter region.

Mutations and polymorphisms in the HNF-4 $\alpha$  gene. The twelve exons, flanking introns and minimal promoter region were screened for mutations in 57 unrelated Japanese subjects with early-onset NIDDM/MODY. This analysis revealed one putative mutation (FIG. 14) and seven DNA polymorphisms/variants (Table 11). The putative 5 mutation in exon 4 at codon 127, CGG (Arg) $\rightarrow$ TGG (Trp) (R127W) alters a conserved amino acid that is located in the T-box, a region implicated in receptor dimerization and DNA binding (Lee *et al.*, 1993; Rastinejad *et al.*, 1995; Gronemeyer and Moras, 1995; Jiang and Sladek *et al.*, 1997). The C $\rightarrow$ T substitution in codon 127 results in the loss of a site for the enzyme *Msp* I and digestion of the normal allele generates fragments of 104, 10, 91, and 76 bp, whereas the mutant allele generates fragments of 104 and 167 bp. PCR™-RFLP analysis showed that the R127W mutation was not present in any of 214 unrelated nondiabetic subjects of different ethnic groups (53 Japanese, 53 Chinese, 51 white and 57 African-American).

TABLE 11

15 DNA Polymorphisms/Variants in the Human HNF-4 $\alpha$  Gene in Japanese Subjects

Location	Nucleotide	Substitution	Allele frequency	
			Early-onset	Nondiabetic
			NIDDM/MODY	
Promoter	nt 922	G $\rightarrow$ A	G-0.99, A-0.01	G-1.00, A-0.00
Intron 1A	nt 1364 (+109)	T $\rightarrow$ C	T-0.99, A-0.01	T-1.00, C-0.00
	nt 1486 (-21)	G $\rightarrow$ A	G-0.99, A-0.01	G-0.99, A-0.01
Intron 1C	nt 2218 (-105)	G $\rightarrow$ A	G-0.99, A-0.01	G-1.00, A-0.00
Intron 1B	nt 2420 (+8)	A $\rightarrow$ G	G-0.99, A-0.01	G-0.99, A-0.01
	nt 3142 (-38)	T $\rightarrow$ C	T-0.28, C-0.72	T-0.24, C-0.76
	nt 3175 (-5)	C $\rightarrow$ T	C-0.84, T-0.16	C-0.86, T-0.14

The R127W mutation was present in three of five diabetic members of the J2-21 family, a MODY family characterized by severe microvascular complications (Iwasaki *et*

*al.*, 1988) (FIG. 15). In addition, subject II-2 must be a carrier since she has children with both normal homozygous and heterozygous genotypes. The age at diagnosis of diabetes in two of the four subjects with the R127W mutation was <25 years (subject II-2, 16 years; and subject III-4, 17 years). One of the subjects with the R127W mutation was  
5 diagnosed with diabetes at 90 years of age indicating the variable penetrance of the mutant allele. Another subject, the 12 year-old son of subject III-4, has inherited the mutant allele but is nondiabetic. However, he is not yet beyond the age at risk and may develop diabetes in the future. There are two subjects with diabetes in the J2-21 family who did not inherit the at-risk allele (subjects III-3 and -6). Such etiological  
10 heterogeneity has been noted previously (Bell *et al.*, 1991).

The seven DNA polymorphisms/variants were located in the promoter region and the introns (Table 11, FIG. 13). In subject J2-96 (FIG. 15), there was a G→A substitution at nucleotide 922 in the proximal promoter region which changes the human sequence so  
15 that it more closely resembles the sequence of the mouse gene (FIG. 13). This substitution was not found on screening 53 nondiabetic subjects. Since this substitution does not alter a conserved residue or disrupt the binding site for one of the factors predicted to regulate transcription of the HNF-4 $\alpha$  gene, the inventors believe that it is a rare variant rather than a diabetes-associated mutation. However, further studies are  
20 necessary to distinguish between these two possibilities.

The six substitutions found in introns (Table 11) do not disrupt the conserved GT and AG dinucleotides of the splice donor and acceptor sites, respectively, and are thus unlikely to affect splicing. The substitutions at nucleotides 1486, 2420, 3142 and 3175  
25 were found in both diabetic and nondiabetic Japanese subjects indicating that they are polymorphisms rather than diabetes-associated mutations. The substitutions at nucleotides 1364 and 2218 were found only in two different unrelated subjects with early-onset NIDDM/MODY. The inventors believe that these are rare variants rather than diabetes-associated mutations as they are not near the splice donor and acceptor sites but  
30 are rather in the central portion of the intron.

## EXAMPLE 5

### Hepatic Function in a Family with a Nonsense Mutation (R154X) in HNF 4 $\alpha$ /MODY1 Gene

5

MODY is a genetically heterogeneous monogenic disorder characterized by autosomal dominant inheritance, onset usually before 25 years of age and abnormal pancreatic  $\beta$ -cell function. Mutations in the hepatocyte nuclear factor (HNF)-4 $\alpha$ /MODY1, glucokinase/MODY2 and HNF-1 $\alpha$ /MODY3 genes can cause this 10 form of diabetes. In contrast to the glucokinase and HNF-1 $\alpha$  genes, mutations in the HNF-4 $\alpha$  gene are a relatively uncommon cause of MODY and the inventors' understanding of the MODY1 form of diabetes is based on studies of only a single family, the R-W pedigree. Here the inventors report the identification of another family with MODY1 and the first in which there has been a detailed characterization of hepatic 15 function. The affected members of this family, Dresden-11 have inherited a nonsense mutation, R154X in the HNF-4 $\alpha$  gene and are predicted to have reduced levels of this transcription factor in the tissues in which it is expressed including pancreatic islets, liver, kidney and intestine. Subjects with the R 154X mutation exhibited a diminished insulin secretory response to oral glucose. HNF-4 $\alpha$  plays a central role in tissue-specific 20 regulation of gene expression in the liver including the control of synthesis of proteins involved in cholesterol and lipoprotein metabolism and the coagulation cascade. However, subjects with the R154X mutation showed no abnormalities in lipid metabolism or coagulation except for a paradoxical 3.3-fold increase in serum lipoprotein(a) levels. Nor was there any evidence of renal dysfunction in these subjects. 25 The results suggest that MODY1 is primarily a disorder of  $\beta$ -cell function.

#### 1. Methods

##### *Subjects.*

The study population consisted of members of twelve unrelated families with early-onset NIDDM ascertained through the Department of Internal Medicine III, University Clinic Carl Gustav Carus of the Technical University, Dresden, Germany. Families were selected based on the presence of non-insulin-dependent (type 2) diabetes mellitus (NIDDM) in two or more generations with diagnosis before 35 years of age in at least one subject. Sufficient family data were available to suggest a diagnosis of MODY in nine of these families (*i.e.*, NIDDM in three generations with autosomal dominant inheritance and onset before 25 years of age in at least one affected subject) (Fajans *et al.*, 1994). The remaining three families were classified as having early-onset NIDDM. The average age at diagnosis of diabetes in affected members of these twelve families was  $29.9 \pm 2.8$  years (range, 14-60 years) (mean $\pm$ SEM) and included 18 men and 13 women of whom 12, 12 and 7 were being treated with insulin, oral hypoglycemic agents and diet, respectively. At the time of recruitment, informed consent was obtained from each subject and blood and urine samples were obtained for DNA isolation and clinical testing.

15

*Screening HNF-4 $\alpha$  gene for mutations.*

The minimal promoter region (nucleotides -21 to -459) (Zhong *et al.*, 1994) and 10 exons encoding the HNF-4 $\alpha$  form (Drewes *et al.*, 1996) of HNF-4 $\alpha$  were screened for mutations by polymerase chain reaction (PCR<sup>TM</sup>) amplification and direct sequencing of both strands of the amplified PCR<sup>TM</sup> product as described previously (Yamagata *et al.*, 1996). Sequence changes were confirmed by cloning the PCR<sup>TM</sup> product into pGEM-4Z (Promega, Madison, WI) and sequencing clones derived from both alleles. The sequences of the primers for the amplification and sequencing of the minimal promoter region are P 1,5'-CAAGGATCCAGAAGATTGGC-3' (SEQ ID NO:120), and P2, 20 5'-CGTCCTCTGGGAAGATCTGC-3' (SEQ ID NO:121); the size of the PCR<sup>TM</sup> product is 479 bp. The sequence of the promoter of the human HNF-4 $\alpha$  gene has been deposited 25 in the GenBank database with accession number U72959.

*Linkage analysis.*

Family members were typed with the markers D20S43, D20S89, D20S96, D20S119, D20S169 and D20S424, all of which are tightly linked to the HNF-4 $\alpha$  gene (Stoffel *et al.*, 1996). Tests for linkage were carried out using the haplotype formed from these markers and assuming a recombination frequency between adjacent markers of 5 0.001 with the computer program ILINK (Lathrop *et al.*, 1984; Lathrop and Lalouel, 1984). The frequencies of the haplotypes were estimated from the data. The analysis assumed a disease allele frequency of 0.001 and two liability classes. Liability class 1 included individuals who were 25 years of age with penetrances of 0.00, 0.95 and 0.95 for the normal homozygote, heterozygote and susceptible homozygote, respectively. 10 Liability class 2 included individuals who were <25 years of age with penetrances of 0.00, 0.60 and 0.95 for the normal homozygote, heterozygote and susceptible homozygote, respectively. The affection status of the one subject with impaired glucose tolerance was coded as affected. The maximum expected lod score (ELOD) was determined using the computer program SLINK (Ott, 1989; Weeks *et al.*, 1990).

15

#### *Clinical Studies.*

A standard 75 g oral glucose tolerance test was given to subjects after a 12 h overnight fast. Treatment with insulin and oral hypoglycemic agents was discontinued 12 h and 24 h, respectively, before testing. Blood samples for glucose, insulin, C-peptide 20 and proinsulin were drawn at 0, 30, 60, 90 and 120 min. Fasting blood samples were also drawn for the measurement of insulin, islet cell and glutamic acid decarboxylase (GAD) antibodies, glycosylated hemoglobin (HbA<sub>1c</sub>), lipoprotein(a), apolipoproteins AI, AII, B, CII, CIII and E, cholesterol (total and in VLDL, LDL, HDL, HDL2 and HDL3), triglycerides (total and in VLDL and LDL+HDL), coagulation time (QUICK test) and 25 partial thromboplastin time (PTT), fibrinogen, von Willebrand factor antigen (vWF:Ag), plasminogen activator inhibitor-1 (PAI-1), tissue-type plasminogen activator (tPA), alanine aminotransferase,  $\gamma$ -glutamyl transferase, bilirubin, albumin, total protein, hemoglobin, creatinine, urea, amylase, lipase and uric acid. A urine sample (from a 24-hour collection of urine) was taken for measurements of creatinine and microalbumin.

30

*Assays.*

Blood glucose was measured with a hexokinase method (Boehringer-Mannheim, Mannheim, Germany), plasma insulin and C-peptide by radioimmunoassay (DPC Biermann GmbH, Bad Nauheim, Germany; and C peptide RIA Diagnostic Systems Laboratories, Sinsheim, Germany, respectively), plasma proinsulin by ELISA (DRG Instruments, Marburg, Germany), HbA<sub>1c</sub> by HPLC (DIAMAT Analyzer, Bio-Rad, Munich, Germany), fibrinogen by the Clauss method (Fibrinogen A Kit, Boehringer-Mannheim), PAI-1 by bioimmunoassay and ELISA (TC<sup>®</sup> Actibind PAI-1 and TC<sup>®</sup> PAI-1 ELISA, Technoclone/Immuno GmbH Deutschland, Heidelberg, Germany), tPA by ELISA (TintElize<sup>®</sup> tPA, Biopool AB, Umeå, Sweden), vWF:Ag enzymatically (ELISA Aserachrom<sup>®</sup> vWF, Boehringer-Mannheim), insulin- and GAD-Ab by ELISA and radioimmunoassay (Elias, Freiburg, Germany), islet cell-Ab by an immunofluorescence assay (using a positive sample from EUROIMMUN Immunologie GmbH, Groß Grönau, Germany), coagulation and partial thromboplastin time by the AMAX Analyzer (Munich, Germany). Total cholesterol, cholesterol in VLDL, HDL, LDL+HDL, and HDL3 were measured by the CHOD-PAP, total triglycerides and triglycerides in VLDL and LDL+HDL by the GPO-PAP method using the Ciba Corning 550 Express Clinical Chemistry Analyzer (Boehringer-Mannheim). HDL2-cholesterol was calculated using the formula HDL2=HDL-HDL3. Samples for the measurement of cholesterol, triglycerides in VLDL, HDL, LDL+HDL were prepared by preparative ultracentrifugation using a Beckman Optima tabletop TLX ultracentrifuge with a TLA-120.2 rotor. Serum creatinine, urea, uric acid, total protein, alanine aminotransferase,  $\gamma$ -glutamyl transferase, bilirubin, amylase and urine creatinine were measured using the BM Hitachi 717 Chemistry Analyzer (Boehringer Mannheim). Lipase was measured using the Monarch System (Sigma Germany, Munich, Germany). Apolipoproteins AI, AII and B and urine microalbumin were measured using the Behring-Nephelometer BN II (Behringwerke, Marburg, Germany). Apolipoproteins CIII and E were measured using the Sebia System (Fulda, Germany), apolipoprotein CII using the RID System (WAK, Bad Homburg, Germany).

## 2. Results

### *Identification of a nonsense mutation in the HNF-4 $\alpha$ gene.*

Twelve families with early-onset NIDDM/MODY were ascertained for genetic studies of MODY in subjects of German ancestry. Mutations in the HNF- $\alpha$ /MODY3 gene (Yamagata *et al.*, 1996) were found in three of these families (Kaisaki *et al.*, 1997). The HNF-4 $\alpha$  gene was screened for mutations in one affected subject from the remaining nine families. There was a C→T substitution in codon 154 of exon 4 in the proband (II-4) of family Dresden-11 (FIG. 16) which generated a nonsense mutation CGA (Arg)→TGA (OP) (R154X, FIG. 17). The R154X mutation would result in the synthesis of a truncated protein of 153 amino acids with an intact DNA binding domain but lacking the ligand binding and transactivation domain (Sladek *et al.*, 1990). In addition to this mutation, there was a silent C→T substitution in the codon for Ala58 (GCC/GCT) in one subject which did not cosegregate with MODY/early-onset NIDDM.

The presence of the R154X mutation in other members of the Dresden-11 family was determined by PCRTM amplification and direct sequencing of exon 4. The R154X mutation cosegregated with MODY in the Dresden-11 family (FIG. 16). All diabetic subjects had the R154X mutation as did a 14-year old male (III-2) with impaired glucose tolerance. The at-risk haplotype showed some evidence for linkage with MODY with a lod score of 1.20 at a recombination of 0.00 (the maximum expected lod score in this pedigree is 1.20).

### *Age at diagnosis.*

Three subjects were diagnosed with NIDDM between 15-25 years of age and two others at 28 and 44 years (FIG. 16). The subject, I-1, diagnosed with diabetes at 44 years of age had proliferative retinopathy at the time of diagnosis suggesting that the onset of diabetes had been many years earlier.

### *Clinical severity of diabetes.*

The diabetes in the Dresden-11 family was severe and all the diabetic subjects were treated with either insulin or oral hypoglycemic agents. Subjects with diabetes of long duration (e.g., I-1, II-4) had diabetic complications including proliferative retinopathy, macrovascular disease (coronary heart disease) and peripheral polyneuropathy. Surprisingly, none of the subjects with the R154X mutation had evidence of nephropathy. Thus, the diabetic phenotype of the Dresden-11 family is very similar to that seen in the R-W pedigree (Fajans *et al.*, 1994). None of the subjects in the Dresden-11 family were positive for islet, insulin or GAD antibodies.

10           *Insulin-secretory response.*

Previous studies have shown that prediabetic subjects with a mutation in HNF-4 $\alpha$  exhibit a characteristic defect in the normal pattern of glucose-stimulated insulin secretion as well as abnormalities in other measures of normal  $\beta$ -cell function (Herman *et al.*, 1994; Byrne *et al.*, 1995). The OGTT studies showed a profound reduction in insulin secretion accompanied by diminished C-peptide and proinsulin levels in subjects with the R154X mutation (FIG. 18).

15           *Lipid levels.*

None of the subjects with the R154X mutation showed evidence of secondary hypertriglyceridemia, even though several (I-1, II-4, III-1) had poor metabolic control with HbA<sub>1c</sub> levels of 10.6, 8.8 and 10.1, respectively (Table 12).

**TABLE 12**  
**Clinical Parameters of the Dresden-11 family**

Parameter	Genotype		Reference values
	Normal/Mutant	Normal/Normal (female/male)	
Age at diagnosis (years)	26.40 ± 3.47	--	--
Current age (years)	35.50 ± 7.58	62/41	--
n (females/males)	2/4	1/1	--
BMI (kg/m <sup>2</sup> )	25.21 ± 1.15	41.08/22.86	<25.00
HbA <sub>1c</sub> (%)	8.13 ± 0.78	5.60/5.30	<6.50
Basal insulin (nM)	0.067 ± 0.005	0.080/0.040	0.059-0.253
Basal C-peptide (nM)	0.60 ± 0.08	0.68/0.45	<1.06
Cholesterol (mM), total	4.72 ± 0.41	5.03/5.01	<5.20
in VLDL (mM)	0.79 ± 0.31	0.21/0.70	0.10-1.40
in LDL (mM)	2.86 ± 0.25	3.62/3.34	1.80-5.10
in HDL (mM)	1.17 ± 0.18	1.32/1.26	0.80-2.50
in HDL2 (mM)	0.31 ± 0.06	0.44/0.27	0.10-0.60
in HDL3 (mM)	0.86 ± 0.12	0.88/0.99	0.80-1.90
Triglycerides (mM), total	0.70 ± 0.13	0.65/1.45	0.40-2.80
in VLDL (mM)	0.43 ± 0.13	0.34/1.06	0.10-2.10
in LDL+HDL (mM)	0.28 ± 0.02	0.33/0.47	0.20-0.80
Lipoprotein (a) (mg/l)	816.0 ± 90.4	3.0/6.0	<250.0
ApoB (g/l)	1.38 ± 0.22	1.33/1.38	0.72-1.50
ApoAI (g/l)	1.66 ± 0.16	1.89/2.00	1.12-1.75
ApoAII (g/l)	0.32 ± 0.02	0.290.53	0.30-0.70
ApoE (mg/l)	61.2 ± 12.2	65.0/55.0	13.0-76.0
ApoCII (mg/l)	36.0 ± 5.3	36.0/61.0	7.0-63.0

ApoCIII (mg/l)	26.7 ± 3.7	23.0/36.0	16.0-45.0
General liver and kidney function			
Hemoglobin (mM)	9.7 ± 0.4	9.2/10.8	8.6-12.1
Creatinine (µM)	91.5 ± 5.6	73.0/80.0	<124.0
Urea (mM)	5.6 ± 0.8	6.6/1.0	3.6-8.9
Total protein (g/l)	72.7 ± 1.7	77.2/84.0	65.0-85.0
Albumin (g/l)	38.6 ± 1.0	38.5/43.5	37.0-53.0
Alanine aminotransferase (µmol/(l's))	0.39 ± 0.06	0.39/0.91	0-10-0.67
γ-glutamyl transferase (µmol/(l's))	0.54 ± 0.12	0.55/1.11	0.18-0.83
Bilirubin (µM), total	16.7 ± 5.2	13.7/24.3	1.0-16.0
Uric acid (µM)	249 ± 28	317/359	208-416
Exocrine pancreatic function			
Amylase (U/l)	56.8 ± 6.7	30.0/58.0	17.0-115.0
Lipase (µmole/(l's))	1.22 ± 0.40	0.20/3.00	0.38-3.40
Coagulation parameters			
Coagulation time (%)	117 ± 6	108/125	70-120
Partial thromboplastin time (s)	33 ± 1	29/35	30-40
Fibrinogen (g/l)	3.54 ± 0.23	2.89/3.69	1.50-4.00
Von Willebrand Factor Antigen (%)	103 ± 11	145/115	70-200
PAI-1 (ng/ml), total	36 ± 8	102/40	30-80
tPA (ng/ml)	10.6 ± 1.5	17.2/16.0	2.0-10.0
Urine analysis			
Creatinine (mM)	8.36 ± 0.88	7.96/2.86	4.66-18.00
Microalbumin (mg/24 h)	<2.2	13.5/<2.2	2.2-18.0

Values are means $\pm$ SEM (standard error of means). The two normal subjects are shown with the single values. Reference values are those from the Institute of Clinical Laboratory Diagnostics, University Clinic Carl Gustav Carus, Dresden.

5       *Hepatic and renal function.*

HNF-4 $\alpha$  is expressed in the liver and kidney and as such mutations in HNF-4 $\alpha$  might be expected to affect the normal function of these tissues (Sladek *et al.*, 1990; Cereghini, 1996). In this regard, HNF-4 $\alpha$  regulates the expression of a number of apolipoproteins including AI, AIV, B and CIII (Cereghini, 1996). The serum 10 apolipoprotein levels and lipoprotein fractions were normal in the subjects with the R154X mutation except for lipoprotein(a) levels, which were elevated 3.3-fold (Table 12). Lipoprotein(a) levels have been reported to be elevated in subjects with NIDDM in some studies (Nakagawa *et al.*, 1996; Hirata *et al.*, 1995) but not others (Durlach *et al.*, 1996; Chico *et al.*, 1996). However, an elevation in lipoprotein(a) levels in subjects with 15 HNF-4 $\alpha$  deficiency appears paradoxical as expression of lipoprotein(a) is controlled by HNF-1 $\alpha$  (Wade *et al.*, 1994) which is in turn regulated by HNF-4 $\alpha$  (Cereghini, 1996). Thus, lower lipoprotein(a) levels not higher would be expected in subjects with the R154X mutation. Further studies will be necessary to determine the relationship between 20 lipoprotein(a) levels and mutations in HNF-4 $\alpha$ .

20

HNF-4 $\alpha$  also regulates the expression of albumin, fibrinogen and the coagulation factors VII, VIII, IX and X (Cereghini, 1996; Erdmann and Heim, 1995; Figueiredo and Brownlee, 1995; Naka and Brownlee, 1996; Hung and High, 1996). The serum levels of albumin and fibrinogen and measurements of coagulation time were normal in subjects 25 with the R154X mutation (Table 12). HNF-4 $\alpha$  is also expressed in the kidney although the identity of the target genes in this organ are unknown (Sladek *et al.*, 1990; Cereghini, 1996). The urinary creatinine and microalbumin levels were normal in subjects with the R154X mutation (Table 12) suggesting that renal function was not impaired in subjects with mutations in the HNF-4 $\alpha$  gene.

## EXAMPLE 6

### Diminished Insulin and Glucagon Secretory Responses to Arginine in Nondiabetic Subject with a Mutation in HNF4 $\alpha$ /MODY1 Gene

5

Nondiabetic subjects with the Q268X mutation in the hepatocyte nuclear factor (HNF)-4 $\alpha$ /MODY1 gene have impaired glucose-induced insulin secretion. To ascertain the effects of the nonglucose secretagogue arginine on insulin and glucagon secretion in these subjects, we studied 18 members of the RW pedigree: 7 nondiabetic mutation 10 negative (ND[-]), 7 nondiabetic mutation positive (ND[+]), and 4 diabetic mutation positive (D[+]). We gave arginine as a 5 g bolus followed by a 25 minute infusion at basal glucose concentrations and after glucose infusion to clamp plasma glucose at ~200 mg/dl. The acute insulin response (AIR), the 10-60 minute insulin area under the curve (AUC), and the insulin secretion rate (ISR) were compared as were acute glucagon 15 response (AGR) and glucagon AUC. The ND[+] and D[+] groups had decreased insulin AUC and ISR and decreased glucose potentiation of AIR, insulin AUC, and ISR to arginine administration when compared to the ND[-] group. At basal glucose concentrations, glucagon AUC was greatest for ND[-], intermediate for ND[+], and lowest for D[+] group. During the hyperglycemic clamp there was decreased suppression 20 of glucagon AUC for both ND[+] and D[+] groups compared to the ND[-] group. The decreased ISR to arginine in the ND[+] group compared to the ND[-] group, magnified by glucose potentiation, indicates that HNF-4 $\alpha$  affects the signaling pathway for arginine-induced insulin secretion. The decrease in glucagon AUC and decreased suppression of glucagon AUC with hyperglycemia suggest that mutations in HNF-4 $\alpha$  may lead to  $\alpha$ -cell 25 as well as  $\beta$ -cell secretory defects or to a reduction in pancreatic islet mass.

#### 1. Methods

##### *Subjects*

Eighteen members of the RW pedigree from branches II-2 and II-5, generations 30 III, IV, and V, were studied (Fajans, 1990; Fajans *et al.*, 1994). The study was reviewed

and approved by the Institutional Review Board of the University of Michigan Medical Center, and all subjects and/or parents provided written informed consent. The glycemic status of each subject was determined by oral glucose tolerance test (OGTT) as defined by the National Diabetes Data Group (NDDG) (1979). Each subject was originally typed  
5 with a series of DNA markers on chromosome 20q to determine whether he or she has inherited the extended at-risk haplotype (defined by alleles at the loci ADA, D20S17, D20S79, and D20S4) associated with MODY1 (Bell *et al.*, 1991; Bowden *et al.*, 1992; Cox *et al.*, 1992; Rothschild *et al.*, 1993). When the Q268X mutation in the HNF-4 $\alpha$  gene was shown to be the cause of MODY1 in the RW pedigree (Yamagata *et al.*, 1996a),  
10 subjects were tested directly for this mutation. All the subjects included in this study, except nondiabetic individual GM11626, have been tested for the presence of the Q268X mutation. However, his nondiabetic father, IV-16, was tested and he does not have the Q268X mutation. Based on the OGTT results and the presence or absence of the Q268X mutation or at-risk haplotype, the family members were subdivided into three groups:  
15

Nondiabetic Q268X mutation-negative group (ND[-])

Seven nondiabetic mutation-negative subjects were studied. GM identification numbers (Human Genetic Mutant Cell Repository) as given by Bell *et al.* (1991), RW pedigree generation and person numbers as given by Fajans *et al.* (1994), and age at the  
20 time of study were: GM10085, IV-22, 45 years; GM11429, IV-41, 32 years; GM11626, offspring of IV-16, 17 years; GM10153, offspring of IV-17, 18 years; GM11579, offspring of IV-19, 16 years; GM11331, offspring of IV-21, 21 years; and GM11333, offspring of IV-21, 22 years. Four of these subjects were offspring of diabetic parents (GM10085, GM11429, GM10153, and GM11579).

Nondiabetic Q268X mutation-positive group (ND[+])

This group included seven subjects. Two subjects never had diabetes or impaired glucose tolerance on OGTT: GM11090, offspring of IV-143, 16 years; and GM10668, offspring of IV-141, 16 years. Five subjects has previous abnormalities of glucose tolerance but none had ever had an abnormal fasting plasma glucose or glycosylated  
30

hemoglobin concentration. Two had single diabetic OGTTs 4 and 22 years, respectively, before the study but had numerous normal glucose tolerance tests subsequently: GM10018, IV-168, 25 years; and GM8072, IV-143, 39 years. Three subjects had fulfilled NDDG diagnostic criteria for diabetes by OGTT in the past. Prior to the study they had 5 normal OGTTs on 2, 4 and 5 occasions, over 2, 4 and 4 years, respectively. They were: GM11600, offspring of IV-143, 14 years; GM8759, IV-166, 31 years; and GM8073, offspring of 143, 19 years.

Diabetic Q268X mutation-positive group (D[+])

10 The four subjects in this group ad consistently diabetic OGTTs for 6 or more years or ad mild fasting hyperglycemia (<200 mg/dl) when untreated. They were GM8106, III-35, 59 years; GM7974, IV-141, 43 years; GM8107, IV-165, 26 years; and GM10724, offspring of IV-142, 17 years. Subject GM8106 was treated with tolbutamide between 1958 and 1968 and with chlorpropamide since May, 1995. When untreated, his highest 15 fasting plasma glucose was 160 mg/dl and his highest total glycosylated hemoglobin 9.1% (normal < 6.3%). On 100 mg of chlorpropamide per day, his fasting plasma glucose was 91 mg/dl and glycosylated hemoglobin was 5.3%. Chlorpropamide was discontinued for 26 days before the study and fasting plasma glucose was 99 mg/dl and total glycosylated hemoglobin concentration was 5.8% on the day of the study. Subject 20 GM7974 was treated with diet alone. She had diabetic OGTTs intermittently since 1969; OGTTs were consistently diabetic since 1990. Her fasting plasma glucose was 84 mg/dl and her total glycosylated hemoglobin was 6.9% at the time of the study. Subject GM8107's highest fasting plasma glucose was 192 mg/dl and highest total glycosylated hemoglobin was 9.5% when untreated. When treated with glyburide 1,25 mg daily, she 25 had normal fasting and postprandial plasma glucose concentrations and a total glycosylated hemoglobin of 6.7%. Glyburide was discontinue 11 days before the study. Her fasting plasma glucose concentration was 106 mg/dl and her total glycosylated hemoglobin was 6.9% on the day of the study. Subject GM10725 had been treated with glyburide 2.5 mg twice daily since 1989. Her highest total glycosylated hemoglobin 30 concentration was 9.0%. She discontinued medication 5 days before the study and her

fasting plasma glucose was 158 mg/dl and her total glycosylated hemoglobin was 7.7% at the time of the study.

*Protocol*

5 Subjects were studied in the University of Michigan General Clinical Research Center (CRC). Subjects were admitted to the CRC in the evening and studied in the recumbent position after a 10-12 hour overnight fast. An intravenous sampling catheter was inserted in a retrograde direction in a dorsal vein of the hand and the hand was kept in a wooden box thermostatically heated to 60°C to achieve arterialization of venous  
10 blood. A second catheter for insulin, arginine and glucose administration was inserted into the contralateral antecubital vein. In subjects with fasting hyperglycemia, a small intravenous bolus of human regular insulin (0.007 U/kg or approximately 0.5 U) was given at -50 minutes to lower the plasma glucose to approximately 75 mg/dl.

15 Blood samples for measurement of basal glucose, insulin, C-peptide, and glucagon concentrations were obtained at -30, -20, -10, and 0 minutes. At 0 minutes, arginine was administered. The total arginine dose was calculated as 0.41 gm/kg body weight to a maximum of 30 grams. At time 0, 5 grams of arginine was administered as an IV bolus over 30 seconds and at time 5 minutes, the remaining arginine was infused  
20 with a pump at a constant rate over 25 minutes. Samples were drawn at 2, 3, 5, 7, 10, 20, and 30-minutes for measurement of glucose, insulin, C-peptide, and glucagon. Following the first arginine bolus and infusion, there was a 60 minute washout period. Blood samples for measurement of the same constituents were obtained at 40, 50, 60, 70, 80, and 90 minutes. At 90 minutes, glucose (150 mg/kg) was administered over 30 seconds  
25 and a variable rate infusion of 20% dextrose with 10 mEq KCl/l was begun to clamp the plasma glucose level at 200 mg/dl for the remainder of the study, as determined by frequent bedside blood glucose measurements. Blood samples for the above constituents were obtained at 92, 93, 95, 97, 100, 110, 120, 130, 140, and 150 minutes. At 150 minutes, arginine (0.41 gm/kg, maximum 30 grams) was again administered as a 5 gram  
30 bolus followed after 5 minutes by an infusion over 25 minutes, as previously, and

samples were drawn at 152, 153, 155, 157, 160, 170, 180, 190, 200, 210, 220, 230, and 240 minutes for measurement of glucose, insulin, C-peptide, and glucagon.

#### *Assay procedures*

5 All blood samples were collected on ice and stored at -70°C until assayed. Plasma glucose was measured on a Kodak Ektachem 700 Analyzer using a hexokinase method (intra-assay coefficient of variation [CV] 1.7% at 5.0 mmol and 1.2% at 16.1 mmol). Immunoreactive insulin was measured by double-antibody radioimmunoassay (RIA) (intra-assay CV 6.4%) (Hayashi *et al.*, 1977). C-peptide was measured by a  
10 specific RIA (intra-assay CV 3.9%) (Faber *et al.*, 1978). Glucagon was measured by double-antibody radioimmunoassay (intra-assay CV 3.2%) (Hayashi *et al.*, 1977). All samples were measured in duplicate and their means were used. Samples from individual subjects were measured in a single assay. All assays were performed in the Michigan Diabetes Research and Training Center Chemistry Core laboratory.

15

#### *Data analysis*

Acute insulin responses (AIR), acute C-peptide responses (ACR), and acute glucagon responses (AGR) were calculated as the mean of the 2, 3, 4, and 5 minute hormone levels minus the mean of the -10, -5, and 0 minute hormone levels. Glucose, 20 insulin, C-peptide, and glucagon areas under the curve were calculated with the trapezoidal rule for the time interval 10 to 60 minute when the arginine bolus was administered at time 0 and the arginine infusion began at time 5 minutes. Baseline values, calculated as the mean hormone levels measured at -10, -5, and 0 minutes immediately preceding the arginine bolus, were subtracted from the areas under the curve.  
25 Insulin secretion rates were calculated by deconvolution of C-peptide values (Polonsky *et al.*, 1986). All of these indices of insulin secretion were assessed during arginine administration at baseline glucose levels, during glucose administration, and during arginine administration during the hyperglycemic clamp. Slope of potentiation was calculated as the difference between the AIR or ACR to arginine obtained during the 30 hyperglycemic clamp and at baseline glucose levels divided by the difference between

these two glucose levels (Halter *et al.*, 1979). Results are expressed as means  $\pm$  standard error of the mean. Statistical significance of differences among groups was assessed with chi-square and unpaired t-tests. The primary comparisons of interest were between the ND[-] and ND[+] group. P < 0.05 was defined as the limit of statistical significance.

5

## 2. Results

Eighteen members of the RW Pedigree were studied: Seven non-diabetic mutation negative (ND[-]), seven non-diabetic mutation positive (ND[+]), and four diabetic mutation positive (D[+]) (Table 13). There were no significant differences among groups with regard to gender or age, although D[+] subjects tended to be older. All subjects were non-obese. Fasting glucose and insulin levels did not differ significantly among groups although D[+] subjects tended to have higher glucose levels and lower insulin levels. Fasting C-peptide levels were lower in D[+] subjects compared to ND[-] subjects. Fasting glucagon levels did not differ among groups. Glycosylated hemoglobin concentration did not differ between the two nondiabetic groups, but was higher in the D[+] group.

**Table 13: Characteristics of Subjects from RW Pedigree by Glucose Tolerance and Mutation Status**

Glucose Tolerance Genotype*	Nondiabetic	Nondiabetic	Diabetic
	[-]	[+]	[+]
<b>Number and gender (M/F)</b>	5/2	3/4	1/3
<b>Age (years)</b>	24 ± 4	23 ± 4	36 ± 9
<b>Body Mass Index (kg/m<sup>2</sup>)</b>	25.2 ± 1.5	23.1 ± 1.0	22.5 ± 0.4
<b>Fasting glucose (mg/dl)</b>	91 ± 2	87 ± 2	112 ± 16
<b>Fasting insulin (μU/ml)</b>	10 ± 1	11 ± 2	7 ± 1
<b>Fasting C-peptide (ng/ml)</b>	1.8 ± 0.1**	1.6 ± 0.2	1.3 ± 0.2
<b>Fasting glucagon (pg/ml)</b>	73 ± 6	64 ± 9	77 ± 12
<b>Glycosylated hemoglobin</b>	5.5 ± 0.1**	5.7 ± 0.2**	7.8 ± 0.4

5                   \*[-] = Normal/Normal  
                   [+] = Normal/Q268X Mutation  
                   \*\*p < 0.05 vs. diabetic [+]  
                   All values are mean ± SEM

10                  FIG. 19 demonstrates the protocol and illustrates concentrations of glucose (FIG. 19A), insulin (FIG. 19B), C-peptide (FIG. 19C), and glucagon (FIG. 19D) during the three phases of the study. These were: A) administration of arginine (bolus and infusion) at basal glucose concentrations, B) administration of glucose (bolus and variable rate infusion) to clamp the glucose level at 200 mg/dl, and C) administration of arginine (bolus and infusion) during the hyperglycemic clamp.

15                  Table 14 summarizes average glucose levels; acute insulin responses (AIR) and C-peptide responses (ACR) to arginine; and hormone areas under the curve (AUC) and insulin secretion rate (ISR) measured 10 to 60 minutes following commencement of the three study phases. These are A) administration of arginine at basal glucose concentrations, B) administration of glucose, and C) administration of arginine during the hyperglycemic clamp.

**Table 14: Plasma Concentrations of Glucose, Acute Insulin and C-peptide Responses (AIR and ACR), Areas Under the Curve (AUC 10-60 minutes) for Insulin and C-peptide and Insulin Secretion Rate (ISR) during administration of A) Arginine at basal glucose concentrations (Bolus and Infusion), B) Glucose (Bolus and Infusion) and C) Arginine (Bolus and Infusion) during hyperglycemic clamp.**

5

Period	Group	Nondiabetic (-)	Nondiabetic (+)	Diabetic (+)
		Number	n = 7	n = 4
<b>A.</b> Arginine administration at basal glucose concentration				
	Glucose (mg/dl)*	107 ± 3	102 ± 2	115 ± 15
	AIR (μU/ml)	48 ± 10	70 ± 19	27 ± 7
	ACR (ng/ml)	3.05 ± 0.61	3.25 ± 0.44	2.19 ± 0.55
	AUC <sub>I</sub> (ng/ml)	78.5 ± 7.7	25.6 ± 5.5 <sup>†</sup>	3.5 ± 0.8 <sup>‡§</sup>
	AUC <sub>C</sub> (ng/ml)	205 ± 12	71 ± 9 <sup>†</sup>	38 ± 6 <sup>‡§</sup>
	ISR (μg)	76 ± 6	31 ± 3 <sup>¶</sup>	16 ± 3 <sup>¶</sup>
<b>B.</b> Glucose administration				
	Glucose (mg/dl)*	207 ± 2	207 ± 5	203 ± 7
	AIR (μU/ml)	72 ± 10	63 ± 15	16 ± 6 <sup>¶</sup>
	ACR (ng/ml)	4.03 ± 0.61	2.83 ± 0.54	1.25 ± 0.58 <sup>#</sup>
	AUC <sub>I</sub> (ng/ml)	43.9 ± 6.3	47.1 ± 11.4	16.1 ± 4.1 <sup>¶</sup>
	AUC <sub>C</sub> (ng/ml)	131 ± 12	103 ± 16	61 ± 22 <sup>#</sup>
	ISR (μg)	63 ± 4	51 ± 6	33 ± 2 <sup>¶</sup>
<b>C.</b> Arginine administration during hyperglycemic clamp				
	Glucose (mg/dl)*	198 ± 2	209 ± 7	201 ± 6
	AIR (μU/ml)	271 ± 33	162 ± 36 <sup>**</sup>	50 ± 10 <sup>‡§</sup>
	ACR (ng/ml)	10.33 ± 1.31	5.87 ± 0.72 <sup>¶</sup>	3.21 ± 0.91 <sup>¶</sup>
	AUC <sub>I</sub> (ng/ml)	628 ± 69	149 ± 40 <sup>†</sup>	25 ± 7 <sup>‡§</sup>
	AUC <sub>C</sub> (ng/ml)	739 ± 52	209 ± 40 <sup>†</sup>	109 ± 42 <sup>‡</sup>
	ISR (μg)	276 ± 18	101 ± 19 <sup>†</sup>	54 ± 16 <sup>‡</sup>

\* mean for period 10-60 minutes

All values are mean  $\pm$  SEM

5 \*\* p  $\leq$  0.05

" p  $\leq$  0.01

<sup>†</sup> p  $\leq$  0.001, ND[+] vs ND[-]

# p < 0.05

\* p < 0.01

<sup>‡</sup> p < 0.001, D[+] vs ND[-]

§ p < 0.05 D[+] vs ND[+]

### *Effects of Arginine and Glucose on Insulin Secretion*

#### Administration of Arginine at Basal Glucose Concentrations

10

At baseline, glucose levels did not differ among the groups (Table 13). After the 5 g arginine bolus, AIR and ACR did not differ among groups but tended to be lower for the D[+] group (Table 14). During and after the subsequent arginine infusion, glucose levels were slightly higher at 10, 20, and 30 minute intervals in the ND[-] as compared to 15 the ND[+] group (FIG. 19) but the average glucose levels during the 10-60 minute time interval (Table 14) and the glucose area under the curve (1171  $\pm$  99 vs. 1012  $\pm$  141 mg/dl, respectively, p = 0.37) did not differ. Insulin and C-peptide levels rose to a peak at 30 minutes in the ND[-] group but were markedly decreased in both the ND[+] and D[+] groups (FIG. 19). The insulin area under the curve (AUC<sub>I</sub>) and C-peptide area under the 20 curve (AUC<sub>C</sub>) were significantly reduced in ND[+] group compare to ND[-] group (Table 14). They were further reduced in D[+] group compared to the ND[+] group (Table 14). ISR was significantly reduced in ND[+] compared to ND[-] subjects and further reduced in D[+] compared to ND[+] subjects (Table 14).

25

#### Administration of Glucose

Glucose levels did not differ among the groups during the bolus and the variable rate glucose infusion (Table 14). AIR and ACR to glucose did not differ between the ND[+] and ND[-] groups but were significantly reduced in the D[+] group compared to the ND[-] group (FIG. 19, Table 14). AUC<sub>I</sub>, AUC<sub>C</sub>, and ISR during the glucose infusion 30 did not differ between the ND[-] and ND[+] groups (Table 14). They were reduced in the D[+] group compared to the ND[-] group (Table 14).

*Administration of Arginine during the Hyperglycemic Clamp*

Glucose levels did not differ among the groups during the variable rate glucose infusion and second arginine bolus and infusion (Table 14). At hyperglycemic plasma glucose levels, as compared to euglycemic levels, AIR and ACR to arginine, and  $AUC_l$ , 5  $AUC_C$  and ISR were enhanced and differences among groups were greatly magnified (FIG. 19, Table 14). All indices of insulin secretion were significantly reduced in the ND[+] group compare to the ND[-] group and there was a further reduction in the D[+] group (Table 14).

10 FIG. 20A and FIG. 20B demonstrates the slopes of potentiation for insulin and C-peptide, respectively. Glucose potentiation of arginine-stimulated insulin secretion was reduced in both the ND[+] ( $0.80 \pm 0.18$ ) and D[+] ( $0.24 \pm 0.04$ ) groups compared to the ND[-] group ( $2.12 \pm 0.25$ ,  $p < 0.001$ ). The insulin slope of potentiation was also reduced in D[+] group compared to ND[+] group ( $p < 0.05$ ). Glucose potentiation of arginine-stimulated C-peptide secretion was also reduced in the ND[+] ( $0.02 \pm 0.00$ ) and D[+] 15 ( $0.01 \pm 0.00$ ) groups compared to the ND[-] group ( $0.07 \pm 0.01$ ,  $p < 0.01$ ).

*Effects of Arginine on Plasma Glucagon Concentrations*

At baseline, glucagon levels did not differ among groups (Table 13). Acute 20 glucagon responses to the 5 g bolus of arginine administered at basal glucose concentrations did not differ significantly among ND[-], ND[+], and D[+] groups ( $104 \pm 19$ ,  $92 \pm 16$ , and  $82 \pm 23$  pg/ml, respectively). On the other hand, the glucagon area under the curve (10-60 minutes) during and following the arginine infusion at basal glucose concentrations was reduced in D[+] compared to ND[-] subjects ( $4,778 \pm 1,087$  vs.  $7,549 \pm 639$  pg/ml,  $p < 0.05$ ). ND[+] subjects showed intermediated volumes ( $5,772 \pm 734$  pg/ml;  $p = 0.09$  vs. ND[-] group). During the hyperglycemic clamp there were no 25 significant differences among glucagon areas under the curve for any of the groups ( $4,237 \pm 406$ ,  $3,963 \pm 508$ , and  $2,941 \pm 568$  pg/ml, for ND[-], ND[+] and D[+], respectively). To assess the impact of glucose infusion on the glucagon response to arginine in the three

study groups, the inventors assessed the differences in glucagon area under the curve between the euglycemic and hyperglycemic periods. Decreases in glucagon areas induced by the hyperglycemic clamp between the first and the second arginine infusion were  $3312 \pm 404$ ,  $1809 \pm 387$ , and  $1836 \pm 535$  pg/ml for the ND[-], ND[+] and D[+] groups,  
5 respectively ( $p < 0.02$  ND[-] vs. ND[+].

## EXAMPLE 7

### MODY Due to Mutations in the HNF-4 $\alpha$ Binding Site in the HNF-1 $\alpha$ Gene Promoter

10

Recent studies have shown that mutations in the transcription factor hepatocyte nuclear factor (HNF)-1 $\alpha$  are the cause of one form of maturity-onset diabetes of the young, MODY3. These studies have identified mutations in the mRNA and protein coding regions of this gene that result in the synthesis of an abnormal mRNA or protein.

15 Here, the inventors report an Italian family in which an A→C substitution at nucleotide -58 of the promoter region of the HNF-1 $\alpha$  gene cosegregates with MODY. This mutation is located in a highly conserved region of the promoter and disrupts the binding site for the transcription factor HNF-4 $\alpha$ , mutations in the gene encoding HNF-4 $\alpha$  being another cause of MODY (MODY1). This result demonstrates that decreased levels of HNF-1 $\alpha$   
20 *per se* can cause MODY. Moreover, it indicates that both the promoter and coding regions of the HNF-1 $\alpha$  gene should be screened for mutations in subjects thought to have MODY because of mutations in this gene.

## 1. Method

### *Subjects*

The MODY family Italy-1 was ascertained through the diabetes clinic of Santo Spirito's Hospital. Affection status was determined using criteria of the National Diabetes Data Group. The affection status of unaffected family members was defined as normal or impaired based on the results of a standard 75 g OGTT. This study had institutional approval and all subjects gave informed consent.

### *Linkage analysis*

Family members were genotyped with the markers D12S321, D12S76 and UC-39 all of which are tightly linked to the HNF-1 $\alpha$  gene (MODY3) (Yamagata *et al.*, 1996). The forward and reverse primers for the polymorphic sequence tagged site (STS) UC-39 are 5'-GCAACAGAGCAAGACTCCATCTCA-3' (SEQ ID NO:122) and 5'-GAGTTAATGGAAGAACTAAC-3' (SEQ ID NO:123) respectively, and the PCR included initial denaturation at 94°C for 5 min and 35 cycles of denaturation at 94°C for 1 min, annealing at 63°C for 1 min and extension at 72°C for 1 min with a final extension at 72°C for 10 min. The forward primer was labeled with  $^{32}\text{P}$  and the MgCl<sub>2</sub> concentration in the reaction was 1.0 mM. The PCR was carried out in a GeneAmp 9600 PCR System (Perkin Elmer, Norwalk, CT). The PCR products were separated by electrophoresis on a 5% polyacrylamide sequencing gel and visualized by autoradiography. Tests for linkage were carried out using the haplotype formed from D12S321, D12S76 and UC-39 and assuming a recombination frequency between adjacent markers of 0.001 with the computer program MLINK from the LINKAGE package (version 5.1) (Lathrop *et al.*, 1985). The frequencies of the haplotypes were estimated from the data. The analysis assumed a disease allele frequency of 0.001 and two liability classes. Liability class 1 included individuals whose age was  $\geq 25$  years of age with penetrances of 0.00, 0.95 and 0.95 for the normal homozygote, heterozygote and susceptible homozygote, respectively. Liability class 2 included individuals  $< 25$  years of age with penetrances of 0.00, 0.50 and 0.95 for the normal homozygote, heterozygote and

susceptible homozygote, respectively. The affection status of the one subject with impaired glucose tolerance was coded as unknown.

#### *Identification of mutations*

5        Each exon and minimal promoter region of the HNF-1 $\alpha$  gene of subjects II-5 and III-1 were screened for mutations as described previously (Yamagata *et al.*, 1996; Kaisaki *et al.*, 1997). The mutation was confirmed by cloning the PCR product into pGEM-4Z and sequencing clones derived from both alleles. The presence of the mutation in other family members and unrelated nondiabetic subjects was tested by PCR amplification of  
10      the proximal promoter region and direct sequencing.

## **2. Results**

### *Linkage studies*

15      The NIDDM in the pedigree Italy-1 has the clinical features of MODY including autosomal dominant inheritance and age at diagnosis <25 years in multiple family members (Fig. 21). The six affected members are treated with either insulin (individuals II-1, II-5 and III-9) or oral hypoglycemic agents (II-7, III-1 and III-2). The three subjects on insulin therapy showed evidence of diabetic complications including retinopathy (II-1 and II-5) and nephropathy (III-9). One member of this pedigree, III-6, has impaired  
20      glucose tolerance.

The polymorphic markers D12S321, D12S76 and UC-39 which are closely linked to the HNF-1 $\alpha$  gene (order: cen - D12S321 - D12S76 - HNF-1 $\alpha$  - UC-39 - qter) were typed in this family. The haplotype 3-3-7 co-segregated with MODY with no obligate recombinants (Fig. 21). One subject with IGT (age, 18 years) also inherited this haplotype as did two unaffected young women, individuals III-5 and III-13, of 21 and 14 years of age, respectively. These three subjects may be at risk of developing diabetes in the future. The LOD score in this family was 1.28 at a recombination fraction of 0.00. Although this LOD score does not meet formal criteria for establishing linkage (ie. the LOD score is

<3.0), the p-value associated with the evidence for linkage is 0.008 which is sufficient to justify a search for mutations in the HNF-1 $\alpha$  gene.

*Mutation screening.*

5 Two diabetic subjects, II-5 and III-1, were screened for mutations in the HNF-1 $\alpha$  gene. No mutations were found on screening the mRNA/protein coding regions, exons 1-10, although the subjects were heterozygous for several previously described polymorphisms (Yamagata et al., 1996). Since no mutations were found in the coding region of the HNF-1 $\alpha$  gene, the proximal promoter region was screened. This analysis  
10 revealed that both affected subjects were heterozygous for an A→C substitution at nucleotide -58 which is located in a highly conserved region of the promoter of the HNF-1 $\alpha$  gene that includes the binding site for HNF-4 $\alpha$  (FIG. 22) (Tian and Schibler et al., 1991; Kuo et al., 1992). Since this mutation does not lead to gain or loss of a site for a restriction endonuclease, it was tested for by PCR amplification and direct sequencing.  
15 The A→C substitution at nucleotide -58 co-segregated with the at-risk haplotype in the Italy-1 pedigree (FIG. 21) and was not present in a sample of 50 unrelated white subjects implying that it is the mutation responsible for MODY in this family.

## EXAMPLE 8

20 **Mutation in HNF-1 $\beta$  associated with MODY**

HNF-1 $\alpha$  and HNF-4 $\alpha$  are members of a complex transcriptional regulatory network which includes other homeodomain proteins and nuclear receptors as well as members of the forkhead/winged helix and leucine zipper CCAAT/enhancer binding  
25 protein families (Cereghini, 1996). The inventors have screened two other members of this network, HNF-1 $\beta$  (Mendel et al., 1991a; De Simone et al., 1991; Rey-Campos et al., 1991; Bach and Yaniv, 1993) and the bifunctional protein dimerization cofactor of HNF-1 (DCoH)/pterin-4 -carbinolamine dehydratase (PCBD) (Mendel et al., 1991b; Citron et al., 1992) for mutations in Japanese subjects with MODY. No diabetes-associated mutations were found in DCoH. However, the inventors found one subject  
30

with a nonsense mutation, R177X, in HNF-1 $\beta$  which co-segregated with early-onset diabetes. The identification of mutations in three members of the HNF-family of transcription factors indicates the importance of this regulatory network in the maintenance of glucose homeostasis.

5

## 1. Methods

### *Study population.*

The study population consisted of 57 unrelated Japanese subjects attending the Diabetes Clinic of Tokyo Women's Medical College who were diagnosed with NIDDM before 25 years of age and/or who were members of families in which NIDDM was present in three or more generations: age at diagnosis,  $20.1 \pm 7.5$  years (mean  $\pm$  SE); male/female, 31/26; and treatment, insulin - 36, oral hypoglycemic agents - 10, and diet - 11. These subjects had been screened for mutations in the HNF-1/MODY3 gene and all were negative for mutations in this gene (Lazzaro *et al.*, 1992). Thirty-two of the subjects met strict criteria for a diagnosis of MODY (*i.e.*, NIDDM in at least three generations with autosomal dominant transmission and diagnosis before 25 years of age in at least one affected subject). NIDDM was diagnosed using the criteria of the World Health Organization (Bennett, 1994). At the time of recruitment, informed consent was obtained from each subject and a blood sample was taken for DNA isolation. Fifty-three unrelated nondiabetic Japanese subjects were tested for each nucleotide substitution and mutation to determine if the sequence change was a polymorphism or disease-associated mutation.

### *Pedigree J2-20.*

The proband (subject III-2, FIG. 25) presented with glucosuria at 10 years of age and was hospitalized. She was diagnosed with diabetes and treated with insulin for two days and then with diet only for two years. At 12 years of age, she resumed insulin therapy (28 U/day). She came to clinical attention again at 21 years because of a pyelonephritis and poorly controlled diabetes. At 23 years of age, she was admitted to the hospital of Tokyo Women's Medical College because of blurred vision. Her urine C-peptide levels at this time were 3.2 g/day (normal,  $50 \pm 25$  g/day) indicating low

insulin secretory capacity. Despite persistent high blood glucose levels, she had no history of ketosis. The subject was diagnosed with NIDDM based on her clinical course. Subject III-3 presented with general fatigue at 15 years of age. He had gained 15 kg during the previous three months and his weight at the time of presentation was 75 kg.

5       He was diagnosed with diabetes and was treated first with insulin and then diet and exercise. He was well controlled when he maintained his weight at 60 kg. At 18 years of age, he had gained weight again and insulin treatment was initiated. His urinary C-peptide at this time was 57.5 g/day with fasting C-peptide and glucose levels of 2.4 ng/ml and 106 mg/dl, respectively. There was no history of ketosis and he was

10      diagnosed with NIDDM. He presently shows diminished pancreatic-cell function with no increase in C-peptide levels following administration of glucagon. All individuals shown in FIG. 25 were invited to participate in this study but many declined to do so.

*Isolation and partial sequence of human HNF-1 $\beta$  gene.*

15      The PAC clone 319P12 containing the human HNF-1 $\beta$  gene was isolated from a library (Genome Systems, St. Louis, MO) by screening PAC DNA pools using polymerase chain reaction (PCR<sup>TM</sup>) and the primers vHNFP1 (5'-CCTCATGGAGAACATCCTAAGT-3') (SEQ ID NO:124) and vHNFP2 (5'-AGGGAGTGCACGGCTGAGCTCCTG-3') (SEQ ID NO: 125). The sequences of

20      the exons, flanking introns and promoter region were determined by sequencing PCR<sup>TM</sup> products and appropriate restriction fragments cloned into pGEM<sup>®</sup>-4Z' (Promega, Madison, WI) with an AmpliTaq FS Dye Terminator cycle sequencing kit (Perkin-Elmer, Norwalk, CT) and ABI Prism<sup>TM</sup> 377 DNA sequencer. Primers for PCR<sup>TM</sup> and sequencing were selected using the exon-intron organization of the human HNF-1 $\alpha$  gene (Yamagata *et al.*, 1996a) as a guide since related genes often have similar exon-intron organizations.

25      The partial sequence of the human HNF-1 $\beta$  gene including promoter has been deposited in the GenBank database under accession numbers U90279-90287 and U96079.

*Mutation screening.*

The nine exons, flanking introns and minimal promoter region of the HNF-1 $\beta$  gene were amplified using PCR<sup>TM</sup> and specific primers (Table 17) and the PCR<sup>TM</sup> products were sequenced from both ends as described above. PCR<sup>TM</sup> for exon 1 was carried out using eLONGASE Enzyme<sup>TM</sup> Mix (Life Technologies, Grand Island, NY) with denaturation at 94°C for 1 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 68°C for 1 min, and final extension at 68°C for 10 min. PCR<sup>TM</sup> for exons 2-9 was carried out using Taq DNA polymerase and 1.5 mM MgCl<sub>2</sub> with denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 30 s, and final extension at 72°C for 10 min. The sequence of each mutation was confirmed by cloning the PCR<sup>TM</sup> product into pGEM<sup>®</sup>-T Easy (Promega, Madison, WI) and sequencing clones representing both alleles. Exons 2-4 of the DCoH gene were amplified using Taq DNA polymerase/1.5 mM MgCl<sub>2</sub> and specific primers (Table 16) and sequenced as described above. Exon 1 of the DCoH gene encoding the 5'-untranslated region and the initiating Met was refractory to PCR<sup>TM</sup> amplification and therefore was not screened for mutations. The presence of a specific mutation or polymorphism in other individuals was determined by PCR-RFLP analysis if it resulted in the gain/loss of a site for a restriction endonuclease, or PCR<sup>TM</sup> and direct sequencing if there was no change in a site.

20           *Linkage studies.*

The human HNF-1 $\beta$  (STS WI-7310) and DCoH genes were mapped and confirmed to YACs 969C9 (chromosome 17) (Schuler *et al.*, 1996) and 849H3 (chromosome 10), respectively. The adjacent polymorphic STSs D17S1788 and D10S1688 were tested for linkage with NIDDM in Japanese affected sib pairs (258 and 25 268 possible pairs, respectively). In the genome-wide screen of Mexican American affected sib pairs 23, the HNF-1 $\beta$  and DCoH genes are in the intervals D17S1293-D17S1299 and D10S589-D10S535, respectively (Schuler *et al.*, 1996).

*Transactivation studies of normal and mutant human HNF-1 $\beta$ .*

The construct pcDNA3.1-HNF-1 $\beta$  was prepared by cloning the type A human HNF-1 $\beta$  cDNA (nucleotides 195-2783 inclusive, GenBank Accession No. X58840; SEQ ID NO:128) into pcDNA3.1+ (Invitrogen, Carlsbad, CA). The R177X mutation was  
5 introduced by site-directed mutagenesis (QuikChange<sup>TM</sup> mutagenesis kit; Stratagene, La Jolla, CA) to generate pcDNA3.1-HNF-1 $\beta$ -R177X. The reporter gene construct pGL3-RA was prepared by cloning the promoter of the rat albumin gene, nucleotides -170 to +5 (Ringeisen *et al.*, 1993), into the firefly luciferase reporter vector pGL3-Basic (Promega, Madison, WI). The sequences of all constructs were confirmed. HeLa cells were  
10 transfected for 5 hr using LipofectAMINE<sup>TM</sup> (GIBCO BRL, Gaithersburg, MD) with 500 ng of pGL3-RA, 250 ng of pcDNA3.1-HNF-1 $\beta$  or pcDNA3.1-HNF-1 $\beta$ -R177X, and 25 ng of pRL-SV40 to control for efficiency of transfection. pcDNA3.1+ DNA was added to each transfection so that the final amount of DNA added was 2 g. After 24 h, the transactivation activity of the normal and mutant HNF-1 $\beta$  proteins was measured  
15 using the Dual-Luciferase<sup>TM</sup> Reporter Assay System (Promega, Madison, WI).

## 2. Results

The nine exons, flanking introns and minimal promoter region of the human HNF-1 $\beta$  gene (*TCF2*) which encode all forms of HNF-1 $\beta$  were screened for mutations in  
20 57 unrelated Japanese subjects with MODY. This analysis revealed four nucleotide substitutions, a C T substitution in codon 177 (exon 2) in the proband from family J2-20 which generated a nonsense mutation CGA (Arg) TGA (OP) (R177X) (FIG. 24), an uncommon silent mutation in codon 463 (exon 7) for which one subject was  
25 homozygous, and two polymorphisms in intron 8 (Table 15), neither of which is predicted to affect RNA splicing. The nonsense mutation R177X was not found on screening 53 unrelated non-diabetic Japanese subjects. One nondiabetic subject was heterozygous for the silent mutation in codon 463 (Table 15).

Table 15. Mutations and DNA polymorphisms in human HNF-1 $\beta$  and DCoH genes

Location			Frequency	
Site	Codon	Nucleotide Change	Patients (n=57)	Controls
<b>A. HNF-1<math>\beta</math></b>				
Exon 2	177	CGA(Arg)→TGA (OP)	C-0.99; T-0.01	C-1.00; T-0.00
Exon 7	463	GCC(Ala)→GCT(Ala)	C-0.98; T-0.02	C-0.99; T-0.01
Intron 8	nt 48	Insertion C	C-0.12	C-0.17
Intron 8	nt -22	C→T	C-0.71; T0.29	C-0.68; T-0.32
<b>B. DCoH</b>				
Exon 4	nt 9306	A→G	A-0.82	A-0.80; G-0.20

DNA polymorphisms found in introns are noted relative to the splice donor or acceptor site. nt, nucleotide. In the HNF1- $\beta$  gene the C→T substitution in codon 463 and the C-insertion polymorphism in intorn 8 nt 48, result in the gain of a Dde I site and loss of a Nae I, respectively. In the human DCoH gene (Genbank accession no. L41560, incorporated herein by reference), the nt 9306 is in the region encoding the 3'-untranslated region of DcoH mRNA and is 36 nucleotides after the translation termination codon.

Family J2-20 shows bilineal inheritance of diabetes (FIG. 25). The R177X mutation, which was maternally inherited, is associated with early-onset NIDDM, progression to insulin treatment and severe complications. The earlier age at diagnosis in the proband and her brother may be due to the inheritance of diabetes-susceptibility genes from both parents. The paternal diabetes gene which may potentiate the effect of the HNF-1 $\beta$  mutation is unknown but is not another known MODY gene as mutations were not found in the HNF-1 $\alpha$  and HNF-4 $\alpha$  and glucokinase genes of the proband (Iwasaki, *et al.*, 1997; Furuta *et al.*, 1997; Iwasaki *et al.*, 1995). The proband's older brother had been healthy until developing a common cold and died one week later of diabetic ketoacidosis.

The proband's maternal grandparents, both of whom are deceased, were not known to have diabetes. However, she has a maternal uncle with mild diet-controlled NIDDM diagnosed at 60 years of age. The difference in phenotype between the proband's mother and maternal uncle and the absence of diabetes in the maternal grandparents suggest that  
5 the R177X mutation may represent a new mutation in the proband's mother. The father and two paternal uncles have late-onset NIDDM treated with oral hypoglycemic agents. The proband's paternal grandmother was reported to have had diabetes. The presence of MODY and late-onset NIDDM within the same family is not unusual and has been reported previously (Bell *et al.*, 1991). With respect to the presence of nephropathy in the  
10 subjects with the R177X mutation in HNF-1 $\beta$ , it is interesting to note that HNF-1 $\beta$  is expressed at highest levels in kidney (Mendel *et al.*, 1991a; De Simone *et al.*, 1991; Rey-Campos *et al.*, 1991; Bach and Yaniv, 1993; Lazzaro *et al.*, 1992) and perhaps decreased levels of this transcription factor contribute to renal dysfunction.

15 HNF-1 $\beta$  contains a bipartite DNA binding region consisting of a POU-like element and a homeodomain (Mendel *et al.*, 1991a; De Simone *et al.*, 1991; Rey-Campos *et al.*, 1991; Bach and Yaniv, 1993). The R177X mutation is located at the end of the POU-like domain and generates a protein of 176 amino acids having the NH<sub>2</sub>-dimerization and POU domains (Cereghini, 1996; Mendel *et al.*, 1991a; De Simone *et al.*,  
20 1991; Rey-Campos *et al.*, 1991; Bach and Yaniv, 1993). This truncated protein cannot stimulate transcription of a rat albumin promoter-linked reporter gene and does not inhibit the activity of wild-type HNF-1 $\beta$  (Table 16). This suggests that the R177X mutation represents a loss of function mutation which results in decreased HNF-1 $\beta$  levels and a corresponding reduction in expression of HNF-1 $\beta$  target genes.

Table 16. Transactivation activity of human HNF-1 $\beta$  and R177X mutation.

Construct	Normalized Activity (Firefly Luciferase/ <i>Renilla</i> luciferase)
pcDNA 3.1	3.5 ± 0.5
pc DNA 3.1-HNF-1 $\beta$	25.1 ± 3.2
pc DNA 3.1- R177X	3.8 ± 1.0
pcDNA 3.1-HNF-1 $\beta$ + pcDNA 3.1-R177X	32.2 ± 2.8

The activity of each construct was measured in triplicate and the mean ±SD is  
5 shown. These results are representative of at least two independent experiments.

**Table 17. Sequences of PCR primers used for amplification and sequencing of human HNF-1 (*TCF2*) and DCoH (*PCBD*) genes**

Region	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)
<b>A. HNF-1 (<i>TCF2</i>)</b>			
Promoter	CATGAACCCCGAACAGACTGGTG (SEQ ID NO:90)	GCCTCCAGACACCTGTTACT SEQ ID NO:91	423
Exon 1-1	GGCGATCATGGCAAGTTAGAAG SEQ ID NO:92	TGGGTGAGAGTATGGAAGACC SEQ ID NO:93	392
Exon 1-2	GGGGTTTGCTTGTGAAACTCC SEQ ID NO:94	TTGGTGGAAACCGGGCTTGG SEQ ID NO:95	536
Exon 2	CTCCCCTAGTACCCCTAACCC SEQ ID NO:96	GAGAGGGCAAAGGGTCACTTCAG SEQ ID NO:97	291
Exon 3	AGTGAAGGCTACAGACCCATTATC SEQ ID NO:98	TTCCCTGGGTCTGTGTACTTGC SEQ ID NO:99	365
Exon 4-1	TGTGTTTGGGCCAACGCACCA SEQ ID NO:100	AACCAGATAAGATCCGGTGGC SEQ ID NO:101	381
Exon 4-2	AACCAAGACTCACAGCCTGAACC SEQ ID NO:102	TCACAGGGCAAATGGCTGAAC SEQ ID NO:103	293

	TGCCGAGTCATTGTTCCAGG	CCTCTTATCTTATCAGCTCCAG	276
Exon 5	SEQ ID NO:104	SEQ ID NO:105	
Exon 6	CTGGCTCTTGTGGTCCAAGTCC	GAGTTTGAAAGGAGAACCTACAG	288
	SEQ ID NO:106	SEQ ID NO:107	
Exon 7	ATCCACCTCTCCCTATCCCAG	ACTTCGGAGAAAGTTCAGACC	340
	SEQ ID NO:108	SEQ ID NO:109	
Exon 8	TTTGCCTGTTATGCACCTTG	GCCGAGTCCATGCTTGCCAC	257
	SEQ ID NO:110	SEQ ID NO:111	
Exon 9	CTTGGCTGGTTGAGTTGGGC	TTCCATGACAGCTGCCAGAG	208
	SEQ ID NO:112	SEQ ID NO:113	
B. DCoH ( <i>PCBD</i> )	TAAAGGTTGGAGCCCCCTCTG	TTGTAAGGTGACCCCATCAG	264
Exon 2	SEQ ID NO:114	SEQ ID NO:115	
Exon 3	TTGGTGATGTCAGAACGCC	CAGAATGTTGTCAGAGTTCGC	213
	SEQ ID NO:116	SEQ ID NO:117	
Exon 4	CTCCCTCCTGTTCTAAAGTG	CTGGACTCCCAGTTCAAGTCA SEQ	205
	SEQ ID NO:118	ID NO:119	

Human DCoH is a protein of 104 amino acids (including the initiating methionine) (Thöny *et al.*, 1995). Exons 2-4 which encode amino acids 2-104 were screened for mutations in the 57 unrelated Japanese subjects with MODY described above. The sequences were identical to one another except for an A G polymorphism located in the 3'-untranslated region (Table 15), the frequency of which was not different between MODY and nondiabetic subjects. Thus, mutations in DCoH do not appear to contribute to the development of MODY in Japanese.

The frequency of HNF-1 $\beta$  mutations in the inventors' study population of Japanese subjects with MODY is 2% (1/57) which is the same as for mutations in HNF-4 $\alpha$  (Furuta *et al.*, 1997) whereas the frequency of HNF-1 $\alpha$  mutations is about 8% (Iwasaki, *et al.*, 1997) (the frequency of glucokinase mutations in this sample is unknown). However, genetic variation in HNF-1 $\beta$  or DCoH is unlikely to be a major factor contributing to the more common late-onset NIDDM as there is no evidence for linkage of markers adjacent to these genes with diabetes in Japanese or Mexican American affected sib pairs (Hanis *et al.*, 1996).

The association of a mutation in HNF-1 $\beta$  with diabetes indicates the importance of the HNF-regulatory network in determining pancreatic-cell function. Moreover, HNF-1 $\alpha$  is not able to compensate for the reduction in HNF-1 $\beta$  activity implying that the primary target genes for these transcription factors in pancreatic  $\beta$ -cells are different. The identification of these target genes will provide a better understanding of the molecular mechanisms that determine normal-cell function and may lead to new approaches for treating diabetes.

## EXAMPLE 9

### Elucidation of the Genes Responsible for Additional MODY Disease States

The inventors have identified that various MODY-type diabetes disease states are caused by mutations in various HNF proteins in the diseased individuals. However, the inventors are also aware of families that exhibit classic "MODY" disease states that are not caused by mutations in HNF1 $\alpha$ , HNF1 $\beta$ , or HNF4 $\alpha$ . Therefore, one aspect of this invention is to continue to screen the genetic complement of these families to determine the genes that cause these additional MODY disease states. Such screening can be done in the manner successfully used by the inventors to screen for the causes of MODY1, MODY2, and MODY 3. One of ordinary skill will be able and motivated in view of the teachings of this application, to work towards elucidating genes that, when mutated, cause additional MODY disease states. Once such genes are elucidated, all aspects diagnostic, treatment, and other aspects of the invention will be realizable by those of skill in the art for those additional MODY causations. In order to achieve these aspects of the invention, one will simply have to modify procedures and protocols taught in this specification to be appropriate to the specific gene determined to cause a MODY disease state.

\* \* \*

All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

## REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein  
5 by reference.

Abbondanzo *et al.*, *Breast Cancer Res. Treat.*, 16: 182(#151), 1990.

Allred *et al.*, *Breast Cancer Res. Treat.*, 16: 182(#149), 1990.

An *et al.*, *Proc. Amer. Assn. Canc. Res.*, 36: 82, 1995.

Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor  
10 Press, Cold Spring Harbor, New York, 1988.

Bach, and Yaniv, *EMBO J.*, 12:4229-4242, 1993.

Baichwal and Sugden, *In:* Kucherlapati R, ed. Gene transfer. New York: Plenum Press, pp.  
117-148, 1986.

Barnett *et al.*, *Diabetologia* 20:87-93 (1981).

15 Baumheuter *et al.*, *Genes and Development*, 4:372-379 1990

Bell, *et al.*, *Proc. Natl. Acad. Sci. USA*, 88:1484-1488, 1991.

Bellus, *J. Macromol. Sci. Pure Appl. Chem.*, A31(1): 1355-1376, 1994.

Bennett, *et al.*, "In: Joslin's Diabetes Mellitus, 13<sup>th</sup> ed., Kahn CR, Weir GC Eds.,  
20 Philadelphia, Lea & Febiger, p. 193-200, 1994.

Benvenisty and Neshif, *Proc. Nat'l Acad. Sci. USA*, 83:9551-9555, 1986.

Bittner *et al.*, *Methods in Enzymol.*, 153:516-544, 1987.

Bourguet, *et al.*, *Nature*, 375:377-382 (1995).

Bowden, *et al.*, *Am. J. Hum. Genet.* 50:607-618 (1992).

Bowden, *et al.*, *Diabetes*, 41:88-92 (1992).

25 Brown *et al.*, *Breast Cancer Res. Treat.*, 16: 192(#191), 1990.

Byrne, *et al.*, *Am. J. Physiol.*, 268:E21-27, 1995a.

Byrne, *et al.*, *Diabetes*, 44(6):699-704, 1995b.

Byrne, *et al.*, *J. Clin. Invest.*, 93:1120-1130, 1994.

Cammidge, P. J., *Br. Med. J.*, 2:738-741 (1928).

30 Capaldi *et al.*, *Biochem. Biophys. Res. Comm.*, 76:425, 1977

Cereghini, *et al.*, *FASEB J.*, 10:267-282, 1996.

Chartier, *et al.*, *Gene*, 147:269-272, 1994.

Chen and Okayama, *Mol. Cell Biol.*, 7:2745-2752, 1987.

Chen *et al.*, *Proc. Am. Urol. Assn.*, 153: 267A, 1995.

5 Chen, *et al. Genes & Dev.*, 8:2466-2477 (1994).

Chico, *et al.*, *Diabetes Res. Clin. Pr.*, 33: 105-110, 1996.

Citron, *et al.*, *Proc. Natl. Acad. Sci. USA*, 89:11891-11894, 1992.

Colberre-Garapin *et al.*, *J. Mol. Biol.*, 150: 1, 1981.

Cotton, R. G. H., *Biochem J.*, 263:1-10 (1989).

10 Courtois *et al.*, *Proc. Nat'l. Acad. Sci. USA*, 85:7937-7941 (1988).

Courtois *et al.*; *Science* 238:688-692 (1987).

Cox, *et al.*, *Diabetes*, 41:401-407, 1992.

Davey *et al.*, EPO No. 329 822.

De Simone, *et al.*, *EMBO J.*, 10:1435-1443 1991.

15 Donahue *et al.*, *J. Biol. Chem.*, 269: 8604-8609, 1994

Drewes, *et al.*, *G.U. Molec. Cell. Biol.*, 16:925-931 (1996).

Dubensky *et al.*, *Proc. Nat'l Acad. Sci. USA*, 81:7529-7533, 1984.

Durlach, *et al.*, *Diabetes & Metabolism*, 22:319-323, 1996.

Eaton, *et al.*, *J. Clin. Endocrinol. Metab.*, 51:520-528, 1980.

20 Ellis, L. A. *et al.*, *Nucleic Acids Res.*, 22:2710-2711 (1994).

Emens, *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 89:7300-7304 (1992).

Erdmann and Heim, *J. Biol. Chem.*, 270:22988-22996, 1995.

European Patent Application No. 139,417A

European Patent Application No. 320 308

25 Faber, *et al.*, *Diabetes*, 27(Suppl. 1):170-177, 1978.

Fajans, *et al.*, *Life Sci.*, 55:413-422, 1994.

Fajans, *Diabetes Care*, 13:49-64, 1990 (Erratum 13:910, 1990).

Fajans, S. S., *Diab./Metab. Rev.* 5, 579-606 (1989).

Fechheimer *et al.*, *Proc. Nat'l Acad. Sci. USA*, 84:8463-8467, 1987.

30 Ferkol *et al.*, *FASEB J.*, 7:1081-1091, 1993.

Figueiredo and Brownlee, *J. Biol. Chem.*, 270:11828-11838, 1995.

Frain *et al.* *Cell*, 59:145-157 (1989).

Fraley *et al.*, *Proc. Natl. Acad. Sci. USA*, 76:3348-3352, 1979.

Freifelder, *Physical Biochemistry Applications to Biochemistry and Molecular Biology*,  
5                   2<sup>nd</sup> ed. Wm. Freeman and Co., New York, NY, 1982.

Freshner, Second Edition, Oxford/New York, IRL Press, Oxford University Press, 1992.

Froguel, *et al.*, *N. Engl. J. Med.*, 328:697-702, 1993.

Froguel, *et al.*, *Nature (Lond.)*, 356:162-164, 1992 (Erratum 357:607, 1992).

Frohman, *PCR PROTOCOLS: A GUIDE TO METHODS AND APPLICATIONS*,  
10                   Academic Press, N.Y., 1990.

Furuta, *et al.*, " *Diabetes*, 46:IN PRESS, 1997.

Gefter *et al.*, *Somatic Cell Genet.*, 3: 231-236, 1977.

German *et al.*, 1990 *JBC* 265, 22063-22066

Ghosh and Bachhawat, In: Wu G. and C. Wu ed. Liver diseases, targeted diagnosis and  
15                   therapy using specific receptors and ligands. New York: Marcel Dekker, pp.  
                      87-104, 1991.

Gibbs, and Caskey, *Science* 236: 303-305 (1987).

Gingeras *et al.*, PCT Application WO 88/10315.

Goding, 1986, In *Monoclonal Antibodies: Principles and Practice*, 2d ed., Orlando, Fla.,  
20                   Academic Press, 1986, pp. 60-61, and 71-74.

Gopal, *Mol. Cell Biol.*, 5:1188-1190, 1985.

Graham and van der Eb, *Virology*, 52:456-467, 1973.

Great Britain Patent Application No. 2 202 328

Gronemeyer and Moras, *Nature*, 375:190-191, 1995.

25                   Halter, *et al.*, *J. Clin. Endocrinol. Metab.*, 48:946-954, 1979.

Hanis, *et al.*, *Nature Genet.*, 13:161-166 (1996).

Hansen *et al.*, *Current Opinion in Genetics and Development*, 3:246-253, 1993.

Harland and Weintraub, *J. Cell Biol.*, 101:1094-1099, 1985.

Hayashi, *et al.*, *J. Clin. Endocrinol. Metab.*, 44:681-94, 1977.

30                   Herman, *et al.* *Diabetes* 43, 40-46 (1994) [Errata, *Diabetes* 43:1171 (1994)].

Hess *et al.*, *J. Adv. Enzyme Reg.*, 7:149, 1968.

Hirata, *et al.*, *Diabetologia*, 38:1434-1442, 1995.

Hitzeman *et al.*, *J. Biol. Chem.*, 255:2073, 1980.

Holland *et al.*, *Biochemistry*, 17:4900, 1978.

5 Hung and High, *J. Biol. Chem.*, 271:2323-2331, 1996.

Innis *et al.*, *PCR Protocols*, Academic Press, Inc., San Diego CA, 1990.

Inouye *et al.*, *Nucleic Acids Res.*, 13: 3101-3109, 1985.

Irwin, *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 91:11684-11688 (1994).

Iwasaki, *et al.*, *Diab. Res. & Clin. Pract.*, 4:237-240, 1988.

10 Iwasaki, *et al.*, *Diabetes*, 46:IN PRESS, 1997.

Iwasaki, *et al.* *J. Japan Diab. Soc.*, 39:409-416 (1996).

Iwaski, *et al.*, *Acta Diabetol* 32:17-22, 1995.

Jiang and Sladek, *J. Biol. Chem.*, 272:1218-1225, 1997.

Jiang, *et al.*, *Molec. Cell. Biol.* 15:5131-5143 (1995).

15 Johnson *et al.*, In *BIOTECHNOLOGY AND PHARMACY*, Pezzuto *et al.*, Eds., Chapman and Hall, New York (1993)

Jones, *Genetics*, 85: 12, 1977.

Kaisaki, *et al.*, *Diabetes*, 46:528-535, 1997.

Kaneda *et al.*, *Science*, 243:375-378, 1989.

20 Kato *et al.*, *J. Biol. Chem.*, 266:3361-3364, 1991.

Kingsman *et al.*, *Gene*, 7: 141, 1979.

Klein *et al.*, *Nature*, 327:70-73, 1987.

Kohler and Milstein, *Eur. J. Immunol.*, 6:511-519, 1976.

Kohler and Milstein, *Nature*, 256:495-497, 1975.

25 Kritis, *et al.*, *Gene*, 173:275-280, 1996.

Kuo *et al.*, *Nature* 355:457-461, 1992.

Kuo, *et al.*, *Nature*, 355:457-461, 1990.

Kwoh *et al.*, *Proc. Nat. Acad. Sci. USA*, 86: 1173, 1989.

Kyte and Doolittle, *J. Mol. Biol.*, 157(1):105-132, 1982.

30 Lai, *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 90:10421-10423 (1993).

Laine, *et al.*, *Gene* 147:269-272 (1994).

Lathrop and Lalouel, *Am. J. Hum. Genet.*, 36:460-465, 1984.

Lathrop, *et al.*, *Am J Hum Genet* 37:482-498, 1985

Lathrop, *et al.*, *Proc. Natl. Acad. Sci. USA*, 81:3443-3446, 1984.

5 Lazzaro, *et al.*, *Development*, 114:469-479, 1992.

Leahy, *et al.*, *Diabetologia*, 36:1238-1244, 1993.

Lederman *et al.*, *Lancet* 345, 648, 1995.

Lee, *et al.*, *Science*, 260:1117-1121, 1993.

Lemaigre, *et al.* *Proc. natl. Acad. Sci. U.S.A.* 93, 9460-9464 (1996).

10 Liang and Pardee, *Science*, 257: 967-971, 1992.

Lishanski *et al.*, *Proc. Nat'l. Acad. Sci USA.*, 91:2674-2678 (1994).

Lowry *et al.*, *Cell*, 22: 817, 1980.

Mangelsdorf, *et al.* *Cell*, 83:835-839 (1995).

Matthews, *et al.*, *Diabetologia*, 28:412-419, 1985.

15 Melton, *et al.*, *Nucleic Acids Res.*, 12:7035-7056, (1984).

Mendel and Crabtree, 1991, *JBC* 266:677-680

Mendel, *et al.* *Science* 254, 1762-1767 (1991b).

Mendel, *et al.*, *Genes & Dev.* 5, 1042-1056 (1991a).

20 Menzel, *et al.*, *Diabetes*, 44:1408-1413, 1995.

Miller *et al.*, PCT Application WO 89/06700

Miquerol, L. *et al.* *J. Biol. Chem.*, 269:8944-8951 (1994).

Mok *et al.*, *Gynecol. Oncol.*, 52: 247-252, 1994.

Morgan and Lazarow, *Diabetes*, 12:115-126, 1963.

Mulligan *et al.*, *Proc. Nat'l Acad. Sci. USA*, 78: 2072, 1981.

25 Myers and Maniatis in U.S. Patent No. 4,946,733

Myers and Maniatis, *Cold Spring Harbor Symposium on Quantitative Biology*, Vo. LI,  
pp. 18275-18284 (1986)

Myers and Maniatis, *Science*, 230:1242-1246 (1985).

Naka and Brownlee, *Brit. J. Haematol.*, 92:231-240, 1996.

30 Nakagawa, *et al.*, *Jap. J. Nephrol.*, 38:513-518, 1996.

Nakamura *et al.*, In: *Handbook of Experimental Immunology* (4<sup>th</sup> Ed.), Weir, E., Herzenberg, L.A., Blackwell, C., Herzenberg, L. (eds). Vol. 1, Chapter 27, Blackwell Scientific Publ., Oxford, 1987.

5 Nicolas & Rubenstein, "Retroviral vectors," In: *Vectors: A survey of molecular cloning vectors and their uses*, Rodriguez & Denhardt (eds.), Stoneham: Butterworth, pp. 493-513, 1988.

Nicolau and Sene, "Liposome-mediated DNA transfer in eukaryotic cells," *Biochem. Biophys. Acta*, 721:185-190, 1982.

O'Hare *et al.*, *Proc. Nat'l Acad. Sci. USA*, 78: 1527, 1981.

10 Ohara *et al.*, *Proc. Nat'l Acad. Sci. USA*, 86: 5673-5677, 1989.

Ott, *Proc. Natl. Acad. Sci. USA*, 86:4175-4178, 1989.

PCT Application No. PCT/US87/00880

PCT Application No. PCT/US89/01025

PCT Application No. WO 88/10315

15 Perales *et al.*, *Proc. Natl. Acad. Sci. USA*, 91:4086-4090, 1994.

Polonsky, *et al.*, *J. Clin. Invest.*, 77:98-105, 1986.

Polonsky, *et al.*, *N. Engl. J. Med.* 334, 777-783 (1996).

Potter *et al.*, *Proc. Nat'l Acad. Sci. USA*, 81:7161-7165, 1984.

Quandt, *et al.*, *Nucl. Acids Res.*, 23:4878-4884, 1995.

20 Rastinejad, *et al.*, *Nature*, 375:203-211, 1995.

Remington's Pharmaceutical Sciences 15<sup>th</sup> Edition, pages 1035-1038 and 1570-1580

Renaud, *et al.* *Nature* 378, 681-689 (1995).

Rey-Campos, *et al.*, *EMBO J.*, 10:1445-1457, 1991.

Ridgeway, In: Rodriguez RL, Denhardt DT, ed. *Vectors: A survey of molecular cloning vectors and their uses*. Stoneham: Butterworth, pp. 467-492, 1988.

25 Ringeisen, *et al.*, *J. Biol. Chem.*, 268:25706-25711, 1993.

Rippe *et al.*, *Mol. Cell Biol.*, 10:689-695, 1990.

Rothschild, *et al.*, *Am. J. Hum. Genet.*, 52:110-23, 1993.

Sager *et al.*, *FASEB J.*, 7: 964-970, 1993.

Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2d Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

Santerre *et al.* 1984

Schuler, *et al.*, *Science*, 274:540-546, 1996.

5 Sladek, *et al.*, *Genes & Dev.* 4, 2353-2365 (1990).

Stinchcomb *et al.*, *Nature*, 282: 39, 1979.

Stoffel, M. *et al.* *Proc. natl. Acad. Sci. U.S.A.*, 93:3937-3941 (1996).

Summers *et al.* A MANUAL OF METHODS FOR BACULOVIRUS VECTORS AND  
INSECT CELL CULTURE PROCEDURES, Texas Agriculture Experimental  
10 Station.

Szybalska *et al.*, *Proc. Nat'l Acad. Sci. USA*, 48: 2026, 1962.

Tavaviras, *et al.*, *Mech. Dev.*, 48:67-79, 1994.

Temin, In: *Gene Transfer*, Kucherlapati (ed.), New York: Plenum Press, pp. 149-188,  
1986.

15 Theophilus, *et al.*, *Nucleic Acids Research*, 17:(19):7707-7722. 1989

Thöny, *et al.*, *Biochem. Biophys. Res. Commun.*, 210:966-973, 1995

Tian and Schibler, *Genes Dev* 5:2225-2234, 1991.

Tschemper *et al.*, *Gene*, 10: 157, 1980.

Tur-Kaspa *et al.*, *Mol. Cell Biol.*, 6:716-718, 1986.

20 U.S. Patent No. 3,817,837

U.S. Patent No. 3,850,752

U.S. Patent No. 3,939,350

U.S. Patent No. 3,996,345

U.S. Patent No. 4,215,051

25 U.S. Patent No. 4,275,149

U.S. Patent No. 4,277,437

U.S. Patent No. 4,366,241

U.S. Patent No. 4,554,101

U.S. Patent No. 4,683,195

30 U.S. Patent No. 4,683,202

U.S. Patent No. 4,800,159  
U.S. Patent No. 4,883,750  
U.S. Patent No. 5,262,311  
U.S. Patent No. 5,279,721

5 Van Cauter, *et al.*, *Diabetes*, 41:368-377, 1992.  
Vaxillaire, *et al.*, *Nature Genetics*, 9:418-423, 1995  
Wade, *et al.*, *J. Biol. Chem.*, 269:19757-19765, 1994.  
Wagner *et al.*, *Science*, 260:1510-1513, 1993.  
Wagner *et al.*, *Science*, 260:1510-1513, 1990.

10 Wagner, *et al.* *Nature* 378, 690-697 (1995)  
Walker *et al.*, *Proc. Nat'l Acad. Sci. USA* 89:392-396 1992.  
Wanke *et al.*, *JBC* 1991, 6068-6072  
Ward, *et al.*, *Diabetes*, 37:723-729, 1988.  
Watson *et al.*, *Cancer Res.*, 54: 4598-4602, 1994.

15 Weeks, *et al.*, *Am. J. Hum. Genet.*, 47:A204, 1990.  
Welsh *et al.*, *Nucleic Acids Res.*, 20: 4965-4970, 1992.  
WHO Study Group on Diabetes Mellitus, *Technical Report Series* 727, World Health Organization, Geneva, 1985.

Wigler *et al.*, *Cell*, 11: 223, 1977.  
20 Wigler *et al.*, *Proc. Nat'l Acad. Sci. USA*, 77: 3567, 1980.  
Winter and Perucho, *Proc. Nat'l Acad. Sci USA*, 82:7575-7579 (1985).  
WO 90/07641 filed December 21, 1990  
Wong *et al.*, *Gene*, 10:87-94, 1980.  
Wong *et al.*, *Int. J. Oncol.*, 3: 13-17, 1993.

25 Wu and Wu, *Adv. Drug Delivery Rev.*, 12:159-167, 1993.  
Wu and Wu, *Adv. Drug Delivery Rev.*, 12:159-167, 1993.  
Wu and Wu, *Biochemistry*, 27:887-892, 1988.  
Wu and Wu, *J. Biol. Chem.*, 262:4429-4432, 1987.  
Wu *et al.*, *Genomics*, 4: 560, 1989.

30 Xanthopoulos, *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 88, 3807-3811 (1991).

Yamagata, *et al.*, *Nature*, 384:458-460, 1996a.  
Yamagata, *et al.*, *Nature*, 384:455-458, 1996b.  
Yang *et al.*, *Proc. Nat'l Acad. Sci. USA*, 87:9568-9572, 1990.  
Zhang, *et al.*, *Molec. Cell. Biol.* 14, 4311-4323 (1994).

5

Zhong, *et al.*, *Mol. Cell. Biol.*, 14:7276-7284, 1994.